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(54) Title: **LIGAND**

(57) Abstract: The invention provides a dual-specific ligand comprising a first immunoglobulin variable domain having a first binding specificity and a complementary or non-complementary immunoglobulin variable domain having a second binding specificity.

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## Ligand

The present invention relates to dual specific ligands. In particular, the invention provides a method for the preparation of dual-specific ligands comprising a first immunoglobulin single variable domain binding to a first antigen or epitope, and a second immunoglobulin single variable domain binding to a second antigen or epitope. More particularly, the invention relates to dual-specific ligands wherein binding to at least one of the first and second antigens or epitopes acts to increase the half-life of the ligand *in vivo*. Open and closed conformation ligands comprising more than one binding specificity are described.

## Introduction

The antigen binding domain of an antibody comprises two separate regions: a heavy chain variable domain ( $V_H$ ) and a light chain variable domain ( $V_L$ : which can be either  $V_K$  or  $V_\lambda$ ). The antigen binding site itself is formed by six polypeptide loops: three from  $V_H$  domain (H1, H2 and H3) and three from  $V_L$  domain (L1, L2 and L3). A diverse primary repertoire of V genes that encode the  $V_H$  and  $V_L$  domains is produced by the combinatorial rearrangement of gene segments. The  $V_H$  gene is produced by the recombination of three gene segments,  $V_H$ , D and  $J_H$ . In humans, there are approximately 51 functional  $V_H$  segments (Cook and Tomlinson (1995) *Immunol Today*, 16: 237), 25 functional D segments (Corbett *et al.* (1997) *J. Mol. Biol.*, 268: 69) and 6 functional  $J_H$  segments (Ravetch *et al.* (1981) *Cell*, 27: 583), depending on the haplotype. The  $V_H$  segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the  $V_H$  domain (H1 and H2), whilst the  $V_H$ , D and  $J_H$  segments combine to form the third antigen binding loop of the  $V_H$  domain (H3). The  $V_L$  gene is produced by the recombination of only two gene segments,  $V_L$  and  $J_L$ . In humans, there are approximately 40 functional  $V_K$  segments (Schäble and Zachau (1993) *Biol. Chem. Hoppe-Seyler*, 374: 1001), 31 functional  $V_\lambda$  segments (Williams *et al.* (1996) *J. Mol. Biol.*, 264: 220; Kawasaki *et al.* (1997) *Genome Res.*, 7: 250), 5 functional  $J_K$  segments (Hieter *et al.* (1982) *J. Biol. Chem.*, 257: 1516) and 4 functional  $J_\lambda$  segments (Vasicek

and Leder (1990) *J. Exp. Med.*, 172: 609), depending on the haplotype. The  $V_L$  segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the  $V_L$  domain (L1 and L2), whilst the  $V_L$  and  $J_L$  segments combine to form the third antigen binding loop of the  $V_L$  domain (L3). Antibodies selected from this primary repertoire are believed to be sufficiently diverse to bind almost all antigens with at least moderate affinity. High affinity antibodies are produced by "affinity maturation" of the rearranged genes, in which point mutations are generated and selected by the immune system on the basis of improved binding.

Analysis of the structures and sequences of antibodies has shown that five of the six antigen binding loops (H1, H2, L1, L2, L3) possess a limited number of main-chain conformations or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, 196: 901; Chothia *et al.* (1989) *Nature*, 342: 877). The main-chain conformations are determined by (i) the length of the antigen binding loop, and (ii) particular residues, or types of residue, at certain key position in the antigen binding loop and the antibody framework. Analysis of the loop lengths and key residues has enabled us to predict the main-chain conformations of H1, H2, L1, L2 and L3 encoded by the majority of human antibody sequences (Chothia *et al.* (1992) *J. Mol. Biol.*, 227: 799; Tomlinson *et al.* (1995) *EMBO J.*, 14: 4628; Williams *et al.* (1996) *J. Mol. Biol.*, 264: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, 263: 800; Shirai *et al.* (1996) *FEBS Letters*, 399: 1).

Bispecific antibodies comprising complementary pairs of  $V_H$  and  $V_L$  regions are known in the art. These bispecific antibodies must comprise two pairs of  $V_H$  and  $V_L$ s, each  $V_H/V_L$  pair binding to a single antigen or epitope. Methods described involve hybrid hybridomas (Milstein & Cuello AC, *Nature* 305:537-40), minibodies (Hu *et al.*, (1996) *Cancer Res* 56:3055-3061;), diabodies (Holliger *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90, 6444-6448; WO 94/13804), chelating recombinant antibodies (CRAbs; (Neri *et al.*, (1995) *J. Mol. Biol.* 246, 367-373), biscFv (e.g. Atwell *et al.*, (1996) *Mol. Immunol.* 33, 1301-1312), "knobs in holes" stabilised antibodies (Carter *et al.*, (1997) *Protein Sci.* 6, 781-

788). In each case each antibody species comprises two antigen-binding sites, each fashioned by a complementary pair of  $V_H$  and  $V_L$  domains. Each antibody is thereby able to bind to two different antigens or epitopes at the same time, with the binding to EACH antigen or epitope mediated by a  $V_H$  and its complementary  $V_L$  domain. Each of these techniques presents its particular disadvantages; for instance in the case of hybrid hybridomas, inactive  $V_H/V_L$  pairs can greatly reduce the fraction of bispecific IgG. Furthermore, most bispecific approaches rely on the association of the different  $V_H/V_L$  pairs or the association of  $V_H$  and  $V_L$  chains to recreate the two different  $V_H/V_L$  binding sites. It is therefore impossible to control the ratio of binding sites to each antigen or epitope in the assembled molecule and thus many of the assembled molecules will bind to one antigen or epitope but not the other. In some cases it has been possible to engineer the heavy or light chains at the sub-unit interfaces (Carter *et al.*, 1997) in order to improve the number of molecules which have binding sites to both antigens or epitopes but this never results in all molecules having binding to both antigens or epitopes.

There is some evidence that two different antibody binding specificities might be incorporated into the same binding site, but these generally represent two or more specificities that correspond to structurally related antigens or epitopes or to antibodies that are broadly cross-reactive.. For example, cross-reactive antibodies have been described, usually where the two antigens are related in sequence and structure, such as hen egg white lysozyme and turkey lysozyme (McCafferty *et al.*, WO 92/01047) or to free hapten and to hapten conjugated to carrier (Griffiths AD *et al.* *EMBO J* 1994 13:14 3245-60). In a further example, WO 02/02773 (Abbott Laboratories) describes antibody molecules with "dual specificity". The antibody molecules referred to are antibodies raised or selected against multiple antigens, such that their specificity spans more than a single antigen. Each complementary  $V_H/V_L$  pair in the antibodies of WO 02/02773 specifies a single binding specificity for two or more structurally related antigens; the  $V_H$  and  $V_L$  domains in such complementary pairs do not each possess a separate specificity. The antibodies thus have a broad single specificity which encompasses two antigens, which are structurally related. Furthermore natural autoantibodies have been described that are polyreactive (Casali & Notkins, *Ann. Rev. Immunol.* 7, 515-531), reacting with at least two (usually more) different antigens or epitopes that are not structurally related. It

has also been shown that selections of random peptide repertoires using phage display technology on a monoclonal antibody will identify a range of peptide sequences that fit the antigen binding site. Some of the sequences are highly related, fitting a consensus sequence, whereas others are very different and have been termed mimotopes (Lane & Stephen, Current Opinion in Immunology, 1993, 5, 268-271). It is therefore clear that a natural four-chain antibody, comprising associated and complementary  $V_H$  and  $V_L$  domains, has the potential to bind to many different antigens from a large universe of known antigens. It is less clear how to create a binding site to two given antigens in the same antibody, particularly those which are not necessarily structurally related.

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Protein engineering methods have been suggested that may have a bearing on this. For example it has also been proposed that a catalytic antibody could be created with a binding activity to a metal ion through one variable domain, and to a hapten (substrate) through contacts with the metal ion and a complementary variable domain (Barbas et al., 1993 Proc. Natl. Acad. Sci USA 90, 6385-6389). However in this case, the binding and catalysis of the substrate (first antigen) is proposed to require the binding of the metal ion (second antigen). Thus the binding to the  $V_H/V_L$  pairing relates to a single but multi-component antigen.

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Methods have been described for the creation of bispecific antibodies from camel antibody heavy chain single domains in which binding contacts for one antigen are created in one variable domain, and for a second antigen in a second variable domain. However the variable domains were not complementary. Thus a first heavy chain variable domain is selected against a first antigen, and a second heavy chain variable domain against a second antigen, and then both domains are linked together on the same chain to give a bispecific antibody fragment (Conrath et al., J. Biol. Chem. 270, 27589-27594). However the camel heavy chain single domains are unusual in that they are derived from natural camel antibodies which have no light chains, and indeed the heavy chain single domains are unable to associate with camel light chains to form complementary  $V_H$  and  $V_L$  pairs.

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Single heavy chain variable domains have also been described, derived from natural antibodies which are normally associated with light chains (from monoclonal antibodies

or from repertoires of domains; see EP-A-0368684). These heavy chain variable domains have been shown to interact specifically with one or more related antigens but have not been combined with other heavy or light chain variable domains to create a ligand with a specificity for two or more different antigens. Furthermore, these single domains have been shown to have a very short *in vivo* half-life. Therefore such domains are of limited therapeutic value.

It has been suggested to make bispecific antibody fragments by linking heavy chain variable domains of different specificity together (as described above). The disadvantage with this approach is that isolated antibody variable domains may have a hydrophobic interface that normally makes interactions with the light chain and is exposed to solvent and may be "sticky" allowing the single domain to bind to hydrophobic surfaces. Furthermore, in the absence of a partner light chain the combination of two or more different heavy chain variable domains and their association, possibly via their hydrophobic interfaces, may prevent them from binding to one in not both of the ligands they are able to bind in isolation. Moreover, in this case the heavy chain variable domains would not be associated with complementary light chain variable domains and thus may be less stable and readily unfold (Worn & Pluckthun, 1998 Biochemistry 37, 13120-7).

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### Summary of the invention

The inventors have described, in their copending international patent application WO 03/002609 as well as copending unpublished UK patent application 0230203.2, dual specific immunoglobulin ligands which comprise immunoglobulin single variable domains which each have different specificities. The domains may act in competition with each other or independently to bind antigens or epitopes on target molecules.

In a first configuration, the present invention provides a further improvement in dual specific ligands as developed by the present inventors, in which one specificity of the ligand is directed towards a protein or polypeptide present *in vivo* in an organism which can act to increase the half-life of the ligand by binding to it.

Accordingly, in a first aspect, there is provided a dual-specific ligand comprising a first immunoglobulin single variable domain having a binding specificity to a first antigen or epitope and a second complementary immunoglobulin single variable domain having a binding activity to a second antigen or epitope, wherein one or both of said antigens or epitopes acts to increase the half-life of the ligand *in vivo* and wherein said first and second domains lack mutually complementary domains which share the same specificity, provided that said dual specific ligand does not consist of an anti-HSA V<sub>H</sub> domain and an anti- $\beta$  galactosidase V<sub>K</sub> domain. Preferably, that neither of the first or second variable domains binds to human serum albumin (HSA).

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Antigens or epitopes which increase the half-life of a ligand as described herein are advantageously present on proteins or polypeptides found in an organism *in vivo*. Examples include extracellular matrix proteins, blood proteins, and proteins present in various tissues in the organism. The proteins act to reduce the rate of ligand clearance from the blood, for example by acting as bulking agents, or by anchoring the ligand to a desired site of action. Examples of antigens/epitopes which increase half-life *in vivo* are given in Annex 1 below.

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Increased half-life is useful in *in vivo* applications of immunoglobulins, especially antibodies and most especially antibody fragments of small size. Such fragments (Fvs, disulphide bonded Fvs, Fabs, scFvs, dAbs) suffer from rapid clearance from the body; thus, whilst they are able to reach most parts of the body rapidly, and are quick to produce and easier to handle, their *in vivo* applications have been limited by their only brief persistence *in vivo*. The invention solves this problem by providing increased half-life of the ligands *in vivo* and consequently longer persistence times in the body of the functional activity of the ligand.

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Methods for pharmacokinetic analysis and determination of ligand half-life will be familiar to those skilled in the art. Details may be found in *Kenneth, A et al*: Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists and in *Peters et al*, Pharmacokinetic analysis: A Practical Approach (1996). Reference is also made to "Pharmacokinetics", M Gibaldi & D Perron, published by Marcel Dekker, 2<sup>nd</sup> Rev. ex

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edition (1982), which describes pharmacokinetic parameters such as  $t_{\alpha}$  and  $t_{\beta}$  half lives and area under the curve (AUC).

Half lives ( $t_{\alpha}$  and  $t_{\beta}$ ) and AUC can be determined from a curve of serum concentration of ligand against time. The WinNonlin analysis package (available from Pharsight Corp., Mountain View, CA94040, USA) can be used, for example, to model the curve. In a first phase (the alpha phase) the ligand is undergoing mainly distribution in the patient, with some elimination. A second phase (beta phase) is the terminal phase when the ligand has been distributed and the serum concentration is decreasing as the ligand is cleared from the patient. The  $t_{\alpha}$  half life is the half life of the first phase and the  $t_{\beta}$  half life is the half life of the second phase. Thus, advantageously, the present invention provides a ligand or a composition comprising a ligand according to the invention having a  $t_{\alpha}$  half-life in the range of 15 minutes or more. In one embodiment, the lower end of the range is 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 10 hours, 11 hours or 12 hours. In addition, or alternatively, a ligand or composition according to the invention will have a  $t_{\alpha}$  half life in the range of up to and including 12 hours. In one embodiment, the upper end of the range is 11, 10, 9, 8, 7, 6 or 5 hours. An example of a suitable range is 1 to 6 hours, 2 to 5 hours or 3 to 4 hours.

Advantageously, the present invention provides a ligand or a composition comprising a ligand according to the invention having a  $t_{\beta}$  half-life in the range of 2.5 hours or more. In one embodiment, the lower end of the range is 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 10 hours, 11 hours, or 12 hours. In addition, or alternatively, a ligand or composition according to the invention has a  $t_{\beta}$  half-life in the range of up to and including 21 days. In one embodiment, the upper end of the range is 12 hours, 24 hours, 2 days, 3 days, 5 days, 10 days, 15 days or 20 days. Advantageously a ligand or composition according to the invention will have a  $t_{\beta}$  half life in the range 12 to 60 hours. In a further embodiment, it will be in the range 12 to 48 hours. In a further embodiment still, it will be in the range 12 to 26 hours.

In addition, or alternatively to the above criteria, the present invention provides a ligand or a composition comprising a ligand according to the invention having an AUC



value (area under the curve) in the range of 1 mg.min/ml or more. In one embodiment, the lower end of the range is 5, 10, 15, 20, 30, 100, 200 or 300mg.min/ml. In addition, or alternatively, a ligand or composition according to the invention has an AUC in the range of up to 600 mg.min/ml. In one embodiment, the upper end of the range is 500, 400, 300, 200, 150, 100, 75 or 50 mg.min/ml. Advantageously a ligand according to the invention will have a AUC in the range selected from the group consisting of the following: 15 to 150mg.min/ml, 15 to 100 mg.min/ml, 15 to 75 mg.min/ml, and 15 to 50mg.min/ml.

In a first embodiment, the dual specific ligand comprises two complementary variable domains, i.e. two variable domains that, in their natural environment, are capable of operating together as a cognate pair or group even if in the context of the present invention they bind separately to their cognate epitopes. For example, the complementary variable domains may be immunoglobulin heavy chain and light chain variable domains ( $V_H$  and  $V_L$ ).  $V_H$  and  $V_L$  domains are advantageously provided by scFv or Fab antibody fragments. Variable domains may be linked together to form multivalent ligands by, for example: provision of a hinge region at the C-terminus of each V domain and disulphide bonding between cysteines in the hinge regions; or provision of dAbs each with a cysteine at the C-terminus of the domain, the cysteines being disulphide bonded together; or production of V-CH & V-CL to produce a Fab format; or use of peptide linkers (for example Gly<sub>4</sub>Ser linkers discussed hereinbelow) to produce dimers, trimers and further multimers.

The inventors have found that the use of complementary variable domains allows the two domain surfaces to pack together and be sequestered from the solvent. Furthermore the complementary domains are able to stabilise each other. In addition, it allows the creation of dual-specific IgG antibodies without the disadvantages of hybrid hybridomas as used in the prior art, or the need to engineer heavy or light chains at the sub-unit interfaces. The dual-specific ligands of the first aspect of the present invention have at least one  $V_H/V_L$  pair. A bispecific IgG according to this invention will therefore comprise two such pairs, one pair on each arm of the Y-shaped molecule. Unlike conventional bispecific antibodies or diabodies, therefore, where the ratio of chains used is determinative in the success of the preparation thereof and leads to practical difficulties, the dual specific ligands of the invention are free from issues of chain balance. Chain

imbalance in conventional bi-specific antibodies results from the association of two different  $V_L$  chains with two different  $V_H$  chains, where  $V_L$  chain 1 together with  $V_H$  chain 1 is able to bind to antigen or epitope 1 and  $V_L$  chain 2 together with  $V_H$  chain 2 is able to bind to antigen or epitope 2 and the two correct pairings are in some way linked to one another. Thus, only when  $V_L$  chain 1 is paired with  $V_H$  chain 1 and  $V_L$  chain 2 is paired with  $V_H$  chain 2 in a single molecule is bi-specificity created. Such bi-specific molecules can be created in two different ways. Firstly, they can be created by association of two existing  $V_H/V_L$  pairings that each bind to a different antigen or epitope (for example, in a bi-specific IgG). In this case the  $V_H/V_L$  pairings must come all together in a 1:1 ratio in order to create a population of molecules all of which are bi-specific. This never occurs (even when complementary CH domain is enhanced by "knobs into holes" engineering) leading to a mixture of bi-specific molecules and molecules that are only able to bind to one antigen or epitope but not the other. The second way of creating a bi-specific antibody is by the simultaneous association of two different  $V_H$  chain with two different  $V_L$  chains (for example in a bi-specific diabody). In this case, although there tends to be a preference for  $V_L$  chain 1 to pair with  $V_H$  chain 1 and  $V_L$  chain 2 to pair with  $V_H$  chain 2 (which can be enhanced by "knobs into holes" engineering of the  $V_L$  and  $V_H$  domains), this pairing is never achieved in all molecules, leading to a mixed formulation whereby incorrect pairings occur that are unable to bind to either antigen or epitope.

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Bi-specific antibodies constructed according to the dual-specific ligand approach according to the first aspect of the present invention overcome all of these problems because the binding to antigen or epitope 1 resides within the  $V_H$  or  $V_L$  domain and the binding to antigen or epitope 2 resides with the complementary  $V_L$  or  $V_H$  domain, respectively. Since  $V_H$  and  $V_L$  domains pair on a 1:1 basis all  $V_H/V_L$  pairings will be bi-specific and thus all formats constructed using these  $V_H/V_L$  pairings (Fv, scFvs, Fabs, minibodies, IgGs etc) will have 100% bi-specific activity.

In the context of the present invention, first and second "epitopes" are understood to be epitopes which are not the same and are not bound by a single monospecific ligand. In the first configuration of the invention, they are advantageously on different antigens, one of which acts to increase the half-life of the ligand *in vivo*. Likewise, the first and second antigens are advantageously not the same.

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The dual specific ligands of the invention do not include ligands as described in WO 02/02773. Thus, the ligands of the present invention do not comprise complementary  $V_H/V_L$  pairs which bind any one or more antigens or epitopes co-operatively. Instead, the  
5 ligands according to the first aspect of the invention comprise a  $V_H/V_L$  complementary pair, wherein the V domains have different specificities.

Moreover, the ligands according to the first aspect of the invention comprise  $V_H/V_L$  complementary pairs having different specificities for non-structurally related epitopes or  
10 antigens. Structurally related epitopes or antigens are epitopes or antigens which possess sufficient structural similarity to be bound by a conventional  $V_H/V_L$  complementary pair which acts in a co-operative manner to bind an antigen or epitope; in the case of structurally related epitopes, the epitopes are sufficiently similar in structure that they “fit” into the same binding pocket formed at the antigen binding site of the  $V_H/V_L$  dimer.

15 In a second aspect, the present invention provides a ligand comprising a first immunoglobulin variable domain having a first antigen or epitope binding specificity and a second immunoglobulin variable domain having a second antigen or epitope binding specificity wherein one or both of said first and second variable domains bind to an  
20 antigen which increases the half-life of the ligand *in vivo*, and the variable domains are not complementary to one another.

In one embodiment, binding to one variable domain modulates the binding of the ligand to the second variable domain.

25 In this embodiment, the variable domains may be, for example, pairs of  $V_H$  domains or pairs of  $V_L$  domains. Binding of antigen at the first site may modulate, such as enhance or inhibit, binding of an antigen at the second site. For example, binding at the first site at least partially inhibits binding of an antigen at a second site. In such an embodiment, the  
30 ligand may for example be maintained in the body of a subject organism *in vivo* through binding to a protein which increases the half-life of the ligand until such a time as it becomes bound to the second target antigen and dissociates from the half-life increasing protein.

Modulation of binding in the above context is achieved as a consequence of the structural proximity of the antigen binding sites relative to one another. Such structural proximity can be achieved by the nature of the structural components linking the two or more antigen binding sites, eg by the provision of a ligand with a relatively rigid structure that holds the antigen binding sites in close proximity. Advantageously, the two or more antigen binding sites are in physically close proximity to one another such that one site modulates the binding of antigen at another site by a process which involves steric hindrance and/or conformational changes within the immunoglobulin molecule.

The first and the second antigen binding domains may be associated either covalently or non-covalently. In the case that the domains are covalently associated, then the association may be mediated for example by disulphide bonds or by a polypeptide linker such as  $(\text{Gly}_4\text{Ser})_n$ , where  $n$  = from 1 to 8, eg, 2, 3, 4, 5 or 7.

Ligands according to the invention may be combined into non-immunoglobulin multi-ligand structures to form multivalent complexes, which bind target molecules with the same antigen, thereby providing superior avidity, while at least one variable domain binds an antigen to increase the half life of the multimer. For example natural bacterial receptors such as SpA have been used as scaffolds for the grafting of CDRs to generate ligands which bind specifically to one or more epitopes. Details of this procedure are described in US 5,831,012. Other suitable scaffolds include those based on fibronectin and affibodies. Details of suitable procedures are described in WO 98/58965. Other suitable scaffolds include lipocalin and CTLA4, as described in van den Beuken *et al.*, J. Mol. Biol. (2001) 310, 591-601, and scaffolds such as those described in WO0069907 (Medical Research Council), which are based for example on the ring structure of bacterial GroEL or other chaperone polypeptides.

Protein scaffolds may be combined; for example, CDRs may be grafted on to a CTLA4 scaffold and used together with immunoglobulin  $V_H$  or  $V_L$  domains to form a ligand. Likewise, fibronectin, lipocalin and other scaffolds may be combined.

In the case that the variable domains are selected from V-gene repertoires selected for instance using phage display technology as herein described, then these variable domains can comprise a universal framework region, such that is they may be recognised by a specific generic ligand as herein defined. The use of universal frameworks, generic  
5 ligands and the like is described in WO99/20749. In the present invention, reference to phage display includes the use of both phage and/or phagemids.

Where V-gene repertoires are used variation in polypeptide sequence is preferably located within the structural loops of the variable domains. The polypeptide sequences of either  
10 variable domain may be altered by DNA shuffling or by mutation in order to enhance the interaction of each variable domain with its complementary pair.

In a preferred embodiment of the invention the 'dual-specific ligand' is a single chain Fv fragment. In an alternative embodiment of the invention, the 'dual-specific ligand'  
15 consists of a Fab region of an antibody. The term "Fab region" includes a Fab-like region where two VH or two VL domains are used.

The variable regions may be derived from antibodies directed against target antigens or epitopes. Alternatively they may be derived from a repertoire of single antibody domains  
20 such as those expressed on the surface of filamentous bacteriophage. Selection may be performed as described below.

In a third aspect, the invention provides a method for producing a ligand comprising a first immunoglobulin single variable domain having a first binding specificity and a  
25 second single immunoglobulin single variable domain having a second (different) binding specificity, one or both of the binding specificities being specific for an antigen which increases the half-life of the ligand *in vivo*, the method comprising the steps of:

- (a) selecting a first variable domain by its ability to bind to a first epitope,
- (b) selecting a second variable region by its ability to bind to a second epitope,
- 30 (c) combining the variable domains; and
- (d) selecting the ligand by its ability to bind to said first epitope and to said second epitope.

The ligand can bind to the first and second epitopes either simultaneously or, where there is competition between the binding domains for epitope binding, the binding of one domain may preclude the binding of another domain to its cognate epitope. In one embodiment, therefore, step (d) above requires simultaneous binding to both first and second (and possibly further) epitopes; in another embodiment, the binding to the first and second epitopes is not simultaneous.

The epitopes are preferably on separate antigens.

Ligands advantageously comprise  $V_H/V_L$  combinations, or  $V_H/V_H$  or  $V_L/V_L$  combinations of immunoglobulin variable domains, as described above. The ligands may moreover comprise camelid  $V_{HH}$  domains, provided that the  $V_{HH}$  domain which is specific for an antigen which increases the half-life of the ligand *in vivo* does not bind Hen egg white lysozyme (HEL), porcine pancreatic alpha-amylase or NmC-A; hcg, BSA-linked RR6 azo dye or *S. mutans* HG982 cells, as described in Conrath *et al.*, (2001) JBC 276:7346-7350 and WO99/23221, neither of which describe the use of a specificity for an antigen which increases half-life to increase the half life of the ligand *in vivo*.

In one embodiment, said first variable domain is selected for binding to said first epitope in absence of a complementary variable domain. In a further embodiment, said first variable domain is selected for binding to said first epitope/antigen in the presence of a third variable domain in which said third variable domain is different from said second variable domain and is complementary to the first domain. Similarly, the second domain may be selected in the absence or presence of a complementary variable domain.

The antigens or epitopes targeted by the ligands of the invention, in addition to the half-life enhancing protein, may be any antigen or epitope but advantageously is an antigen or epitope that is targeted with therapeutic benefit. The invention provides ligands, including open conformation, closed conformation and isolated dAb monomer ligands, specific for any such target, particularly those targets further identified herein. Such targets may be, or be part of, polypeptides, proteins or nucleic acids, which may be naturally occurring or synthetic. In this respect, the ligand of the invention may bind the epitope or antigen and act as an antagonist or agonist (eg, EPO receptor agonist). One

skilled in the art will appreciate that the choice is large and varied. They may be for instance human or animal proteins, cytokines, cytokine receptors, enzymes co-factors for enzymes or DNA binding proteins. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, EGF receptor, ENA-78, 5 Eotaxin, Eotaxin-2, Exodus-2, EpoR, FGF-acidic, FGF-basic, fibroblast growth factor-10, FLT3 ligand, Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF- $\beta$ 1, insulin, IFN- $\gamma$ , IGF-I, IGF-II, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin  $\alpha$ , Inhibin  $\beta$ , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotactin, 10 Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein, M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-3 $\alpha$ , MIP-3 $\beta$ , MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor,  $\beta$ -NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, 15 SDF1 $\alpha$ , SDF1 $\beta$ , SCF, SCGF, stem cell factor (SCF), TARC, TGF- $\alpha$ , TGF- $\beta$ , TGF- $\beta$ 2, TGF- $\beta$ 3, tumour necrosis factor (TNF), TNF- $\alpha$ , TNF- $\beta$ , TNF receptor I, TNF receptor II, TNIL-1, TPO, VEGF, VEGF receptor 1, VEGF receptor 2, VEGF receptor 3, GCP-2, GRO/MGSA, GRO- $\beta$ , GRO- $\gamma$ , HCC1, 1-309, HER 1, HER 2, HER 3 and HER 4. Cytokine receptors include receptors for the foregoing cytokines. It will be appreciated 20 that this list is by no means exhaustive.

In one embodiment of the invention, the variable domains are derived from a respective antibody directed against the antigen or epitope. In a preferred embodiment the variable domains are derived from a repertoire of single variable antibody domains.

25

In a further aspect, the present invention provides one or more nucleic acid molecules encoding at least a dual-specific ligand as herein defined. The dual specific ligand may be encoded on a single nucleic acid molecule; alternatively, each domain may be encoded by a separate nucleic acid molecule. Where the ligand is encoded by a single nucleic acid 30 molecule, the domains may be expressed as a fusion polypeptide, in the manner of a scFv molecule, or may be separately expressed and subsequently linked together, for example using chemical linking agents. Ligands expressed from separate nucleic acids will be linked together by appropriate means.

The nucleic acid may further encode a signal sequence for export of the polypeptides from a host cell upon expression and may be fused with a surface component of a filamentous bacteriophage particle (or other component of a selection display system) upon expression.

In a further aspect the present invention provides a vector comprising nucleic acid encoding a dual specific ligand according to the present invention.

10 In a yet further aspect, the present invention provides a host cell transfected with a vector encoding a dual specific ligand according to the present invention.

Expression from such a vector may be configured to produce, for example on the surface of a bacteriophage particle, variable domains for selection. This allows selection of displayed variable regions and thus selection of 'dual-specific ligands' using the method of the present invention.

The present invention further provides a kit comprising at least a dual-specific ligand according to the present invention.

20

Dual-Specific ligands according to the present invention preferably comprise combinations of heavy and light chain domains. For example, the dual specific ligand may comprise a  $V_H$  domain and a  $V_L$  domain, which may be linked together in the form of an scFv. In addition, the ligands may comprise one or more  $C_H$  or  $C_L$  domains. For example, the ligands may comprise a  $C_{H1}$  domain,  $C_{H2}$  or  $C_{H3}$  domain, and/or a  $C_L$  domain,  $C_{\mu 1}$ ,  $C_{\mu 2}$ ,  $C_{\mu 3}$  or  $C_{\mu 4}$  domains, or any combination thereof. A hinge region domain may also be included. Such combinations of domains may, for example, mimic natural antibodies, such as IgG or IgM, or fragments thereof, such as Fv, scFv, Fab or  $F(ab')_2$  molecules. Other structures, such as a single arm of an IgG molecule comprising  $V_H$ ,  $V_L$ ,  $C_{H1}$  and  $C_L$  domains, are envisaged.

30

In a preferred embodiment of the invention, the variable regions are selected from single domain V gene repertoires. Generally the repertoire of single antibody domains is



displayed on the surface of filamentous bacteriophage. In a preferred embodiment each single antibody domain is selected by binding of a phage repertoire to antigen.

In a preferred embodiment of the invention each single variable domain may be selected  
5 for binding to its target antigen or epitope in the absence of a complementary variable region. In an alternative embodiment, the single variable domains may be selected for binding to its target antigen or epitope in the presence of a complementary variable region. Thus the first single variable domain may be selected in the presence of a third complementary variable domain, and the second variable domain may be selected in the  
10 presence of a fourth complementary variable domain. The complementary third or fourth variable domain may be the natural cognate variable domain having the same specificity as the single domain being tested, or a non-cognate complementary domain – such as a “dummy” variable domain.

15 Preferably, the dual specific ligand of the invention comprises only two variable domains although several such ligands may be incorporated together into the same protein, for example two such ligands can be incorporated into an IgG or a multimeric immunoglobulin, such as IgM. Alternatively, in another embodiment a plurality of dual specific ligands are combined to form a multimer. For example, two different dual  
20 specific ligands are combined to create a tetra-specific molecule.

It will be appreciated by one skilled in the art that the light and heavy variable regions of a dual-specific ligand produced according to the method of the present invention may be on the same polypeptide chain, or alternatively, on different polypeptide chains. In the  
25 case that the variable regions are on different polypeptide chains, then they may be linked via a linker, generally a flexible linker (such as a polypeptide chain), a chemical linking group, or any other method known in the art.

In a further aspect, the present invention provides a composition comprising a dual-  
30 specific ligand, obtainable by a method of the present invention, and a pharmaceutically acceptable carrier, diluent or excipient.

Moreover, the present invention provides a method for the treatment and/or prevention of disease using a 'dual-specific ligand' or a composition according to the present invention.

5 In a second configuration, the present invention provides multispecific ligands which comprise at least two non-complementary variable domains. For example, the ligands may comprise a pair of  $V_H$  domains or a pair of  $V_L$  domains. Advantageously, the domains are of non-camelid origin; preferably they are human domains or comprise human framework regions (FWs) and one or more heterologous CDRs. CDRs and  
10 framework regions are those regions of an immunoglobulin variable domain as defined in the Kabat database of Sequences of Proteins of Immunological Interest.

Preferred human framework regions are those encoded by germline gene segments DP47 and DPK9. Advantageously, FW1, FW2 and FW3 of a  $V_H$  or  $V_L$  domain have the  
15 sequence of FW1, FW2 or FW3 from DP47 or DPK9. The human frameworks may optionally contain mutations, for example up to about 5 amino acid changes or up to about 10 amino acid changes collectively in the human frameworks used in the ligands of the invention.

20 The variable domains in the multispecific ligands according to the second configuration of the invention may be arranged in an open or a closed conformation; that is, they may be arranged such that the variable domains can bind their cognate ligands independently and simultaneously, or such that only one of the variable domains may bind its cognate ligand at any one time.

25 The inventors have realised that under certain structural conditions, non-complementary variable domains (for example two light chain variable domains or two heavy chain variable domains) may be present in a ligand such that binding of a first epitope to a first variable domain inhibits the binding of a second epitope to a second variable domain,  
30 even though such non-complementary domains do not operate together as a cognate pair.

Advantageously, the ligand comprises two or more pairs of variable domains; that is, it comprises at least four variable domains. Advantageously, the four variable domains comprise frameworks of human origin.

- 5 In a preferred embodiment, the human frameworks are identical to those of human germline sequences.

The present inventors consider that such antibodies will be of particular use in ligand binding assays for therapeutic and other uses.

10

Thus, in a first aspect of the second configuration, the present invention provides a method for producing a multispecific ligand comprising the steps of:

- a) selecting a first epitope binding domain by its ability to bind to a first epitope,  
b) selecting a second epitope binding domain by its ability to bind to a second  
15 epitope,  
c) combining the epitope binding domains; and  
d) selecting the closed conformation multispecific ligand by its ability to bind to said first second epitope and said second epitope.

- 20 In a further aspect of the second configuration, the invention provides method for preparing a closed conformation multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity, wherein the first and second binding specificities compete for epitope binding such that the closed  
25 conformation multi-specific ligand may not bind both epitopes simultaneously, said method comprising the steps of:

- a) selecting a first epitope binding domain by its ability to bind to a first epitope,  
b) selecting a second epitope binding domain by its ability to bind to a second  
30 epitope,  
c) combining the epitope binding domains such that the domains are in a closed conformation; and

- d) selecting the closed conformation multispecific ligand by its ability to bind to said first second epitope and said second epitope, but not to both said first and second epitopes simultaneously.

5 Moreover, the invention provides a closed conformation multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity, wherein the first and second binding specificities compete for epitope binding such that the closed conformation multi-specific ligand may not bind both epitopes  
10 simultaneously.

An alternative embodiment of the above aspect of the of the second configuration of the invention optionally comprises a further step (b1) comprising selecting a third or further epitope binding domain. In this way the multi-specific ligand produced, whether of open  
15 or closed conformation, comprises more than two epitope binding specificities. In a preferred aspect of the second configuration of the invention, where the multi-specific ligand comprises more than two epitope binding domains, at least two of said domains are in a closed conformation and compete for binding; other domains may compete for binding or may be free to associate independently with their cognate epitope(s).

20 According to the present invention the term 'multi-specific ligand' refers to a ligand which possesses more than one epitope binding specificity as herein defined.

As herein defined the term 'closed conformation' (multi-specific ligand) means that the epitope binding domains of the ligand are attached to or associated with each other,  
25 optionally by means of a protein skeleton, such that epitope binding by one epitope binding domain competes with epitope binding by another epitope binding domain. That is, cognate epitopes may be bound by each epitope binding domain individually but not simultaneously. The closed conformation of the ligand can be achieved using methods  
30 herein described.

"Open conformation" means that the epitope binding domains of the ligand are attached to or associated with each other, optionally by means of a protein skeleton, such that

epitope binding by one epitope binding domain does not compete with epitope binding by another epitope binding domain.

As referred to herein, the term 'competes' means that the binding of a first epitope to its cognate epitope binding domain is inhibited when a second epitope is bound to its cognate epitope binding domain. For example, binding may be inhibited sterically, for example by physical blocking of a binding domain or by alteration of the structure or environment of a binding domain such that its affinity or avidity for an epitope is reduced.

In a further embodiment of the second configuration of the invention, the epitopes may displace each other on binding. For example, a first epitope may be present on an antigen which, on binding to its cognate first binding domain, causes steric hindrance of a second binding domain, or a conformational change therein, which displaces the epitope bound to the second binding domain.

Advantageously, binding is reduced by 25% or more, advantageously 40%, 50%, 60%, 70%, 80%, 90% or more, and preferably up to 100% or nearly so, such that binding is completely inhibited. Binding of epitopes can be measured by conventional antigen binding assays, such as ELISA, by fluorescence based techniques, including FRET, or by techniques such as surface plasmon resonance which measure the mass of molecules.

According to the method of the present invention, advantageously, each epitope binding domain is of a different epitope binding specificity.

In the context of the present invention, first and second "epitopes" are understood to be epitopes which are not the same and are not bound by a single monospecific ligand. They may be on different antigens or on the same antigen, but separated by a sufficient distance that they do not form a single entity that could be bound by a single mono-specific  $V_H/V_L$  binding pair of a conventional antibody. Experimentally, if both of the individual variable domains in single chain antibody form (domain antibodies or dAbs) are separately competed by a monospecific  $V_H/V_L$  ligand against two epitopes then those two epitopes are not sufficiently far apart to be considered separate epitopes according to the present invention.

The closed conformation multispecific ligands of the invention do not include ligands as described in WO 02/02773. Thus, the ligands of the present invention do not comprise complementary  $V_H/V_L$  pairs which bind any one or more antigens or epitopes co-operatively. Instead, the ligands according to the invention preferably comprise non-complementary  $V_H-V_H$  or  $V_L-V_L$  pairs. Advantageously, each  $V_H$  or  $V_L$  domain in each  $V_H-V_H$  or  $V_L-V_L$  pair has a different epitope binding specificity, and the epitope binding sites are so arranged that the binding of an epitope at one site competes with the binding of an epitope at another site.

10

According to the present invention, advantageously, each epitope binding domain comprises an immunoglobulin variable domain. More advantageously, each immunoglobulin variable domain will be either a variable light chain domain ( $V_L$ ) or a variable heavy chain domain  $V_H$ . In the second configuration of the present invention, the immunoglobulin domains when present on a ligand according to the present invention are non-complementary, that is they do not associate to form a  $V_H/V_L$  antigen binding site. Thus, multi-specific ligands as defined in the second configuration of the invention comprise immunoglobulin domains of the same sub-type, that is either variable light chain domains ( $V_L$ ) or variable heavy chain domains ( $V_H$ ). Moreover, where the ligand according to the invention is in the closed conformation, the immunoglobulin domains may be of the camelid  $V_{HH}$  type.

15  
20

In an alternative embodiment, the ligand(s) according to the invention do not comprise a camelid  $V_{HH}$  domain. More particularly, the ligand(s) of the invention do not comprise one or more amino acid residues that are specific to camelid  $V_{HH}$  domains as compared to human  $V_H$  domains.

25

Advantageously, the single variable domains are derived from antibodies selected for binding activity against different antigens or epitopes. For example, the variable domains may be isolated at least in part by human immunisation. Alternative methods are known in the art, including isolation from human antibody libraries and synthesis of artificial antibody genes.

30

The variable domains advantageously bind superantigens, such as protein A or protein L. Binding to superantigens is a property of correctly folded antibody variable domains, and allows such domains to be isolated from, for example, libraries of recombinant or mutant domains.

5

Epitope binding domains according to the present invention comprise a protein scaffold and epitope interaction sites (which are advantageously on the surface of the protein scaffold).

10 Epitope binding domains may also be based on protein scaffolds or skeletons other than immunoglobulin domains. For example natural bacterial receptors such as SpA have been used as scaffolds for the grafting of CDRs to generate ligands which bind specifically to one or more epitopes. Details of this procedure are described in US 5,831,012. Other suitable scaffolds include those based on fibronectin and affibodies. Details of suitable  
15 procedures are described in WO 98/58965. Other suitable scaffolds include lipocalin and CTLA4, as described in van den Beuken *et al.*, J. Mol. Biol. (2001) 310, 591-601, and scaffolds such as those described in WO0069907 (Medical Research Council), which are based for example on the ring structure of bacterial GroEL or other chaperone polypeptides.

20

Protein scaffolds may be combined; for example, CDRs may be grafted on to a CTLA4 scaffold and used together with immunoglobulin V<sub>H</sub> or V<sub>L</sub> domains to form a multivalent ligand. Likewise, fibronectin, lipocalin and other scaffolds may be combined.

25 It will be appreciated by one skilled in the art that the epitope binding domains of a closed conformation multispecific ligand produced according to the method of the present invention may be on the same polypeptide chain, or alternatively, on different polypeptide chains. In the case that the variable regions are on different polypeptide chains, then they may be linked via a linker, advantageously a flexible linker (such as a polypeptide chain),  
30 a chemical linking group, or any other method known in the art.

The first and the second epitope binding domains may be associated either covalently or non-covalently. In the case that the domains are covalently associated, then the association may be mediated for example by disulphide bonds.

5 In the second configuration of the invention, the first and the second epitopes are preferably different. They may be, or be part of, polypeptides, proteins or nucleic acids, which may be naturally occurring or synthetic. In this respect, the ligand of the invention may bind an epitope or antigen and act as an antagonist or agonist (eg, EPO receptor agonist). The epitope binding domains of the ligand in one embodiment have the same  
10 epitope specificity, and may for example simultaneously bind their epitope when multiple copies of the epitope are present on the same antigen. In another embodiment, these epitopes are provided on different antigens such that the ligand can bind the epitopes and bridge the antigens. One skilled in the art will appreciate that the choice of epitopes and antigens is large and varied. They may be for instance human or animal proteins,  
15 cytokines, cytokine receptors, enzymes co-factors for enzymes or DNA binding proteins. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, EGF receptor, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, EpoR, FGF-acidic, FGF-basic, fibroblast growth factor-10, FLT3 ligand, Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF- $\beta$ 1, insulin, IFN- $\gamma$ , IGF-I, IGF-II, IL-1 $\alpha$ , IL-1 $\beta$ ,  
20 IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin  $\alpha$ , Inhibin  $\beta$ , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotoxin, Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein, M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69  
25 a.a.), MIG, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-3 $\alpha$ , MIP-3 $\beta$ , MIP-4, myeloid progenitor inhibitor factor-1 (MPLF-1), NAP-2, Neurturin, Nerve growth factor,  $\beta$ -NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1 $\alpha$ , SDF1 $\beta$ , SCF, SCGF, stem cell factor (SCF), TARC, TGF- $\alpha$ , TGF- $\beta$ , TGF- $\beta$ 2, TGF- $\beta$ 3, tumour necrosis factor (TNF), TNF- $\alpha$ , TNF- $\beta$ , TNF receptor I, TNF receptor II, TNF-1, TPO, VEGF,  
30 VEGF receptor 1, VEGF receptor 2, VEGF receptor 3, GCP-2, GRO/MGSA, GRO- $\beta$ , GRO- $\gamma$ , HCC1, 1-309, HER 1, HER 2, HER 3, HER 4, TACE recognition site, TNF BP-I and TNF BP-II, as well as any target disclosed in Annex 2 or Annex 3 hereto, whether in combination as set forth in the Annexes, in a different combination or individually.



Cytokine receptors include receptors for the foregoing cytokines, e.g. IL-1 R1; IL-6R; IL-10R; IL-18R, as well as receptors for cytokines set forth in Annex 2 or Annex 3 and also receptors disclosed in Annex 2 and 3. It will be appreciated that this list is by no means exhaustive. Where the multispecific ligand binds to two epitopes (on the same or  
5 different antigens), the antigen(s) may be selected from this list.

Advantageously, dual specific ligands may be used to target cytokines and other molecules which cooperate synergistically in therapeutic situations in the body of an organism. The invention therefore provides a method for synergising the activity of two  
10 or more cytokines, comprising administering a dual specific ligand capable of binding to said two or more cytokines. In this aspect of the invention, the dual specific ligand may be any dual specific ligand, including a ligand composed of complementary and/or non-complementary domains, a ligand in an open conformation, and a ligand in a closed conformation. For example, this aspect of the invention relates to combinations of V<sub>H</sub>  
15 domains and V<sub>L</sub> domains, V<sub>H</sub> domains only and V<sub>L</sub> domains only.

Synergy in a therapeutic context may be achieved in a number of ways. For example, target combinations may be therapeutically active only if both targets are targeted by the ligand, whereas targeting one target alone is not therapeutically effective. In another  
20 embodiment, one target alone may provide some low or minimal therapeutic effect, but together with a second target the combination provides a synergistic increase in therapeutic effect.

Preferably, the cytokines bound by the dual specific ligands of this aspect of the invention  
25 are selected from the list shown in Annex 2.

Moreover, dual specific ligands may be used in oncology applications, where one specificity targets CD89, which is expressed by cytotoxic cells, and the other is tumour specific. Examples of tumour antigens which may be targetted are given in Annex 3.

30 In one embodiment of the second configuration of the invention, the variable domains are derived from an antibody directed against the first and/or second antigen or epitope. In a preferred embodiment the variable domains are derived from a repertoire of single

variable antibody domains. In one example, the repertoire is a repertoire that is not created in an animal or a synthetic repertoire. In another example, the single variable domains are not isolated (at least in part) by animal immunisation. Thus, the single domains can be isolated from a naïve library.

5

The second configuration of the invention, in another aspect, provides a multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity. The first and second binding specificities may be the same or  
10 different.

15

In a further aspect, the present invention provides a closed conformation multi-specific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity wherein the first and second binding specificities are capable of competing for epitope binding such that the closed conformation multi-specific ligand cannot bind both epitopes simultaneously.

20

In a still further aspect, the invention provides open conformation ligands comprising non-complementary binding domains, wherein the domains are specific for a different epitope on the same target. Such ligands bind to targets with increased avidity. Similarly, the invention provides multivalent ligands comprising non-complementary binding domains specific for the same epitope and directed to targets which comprise multiple copies of said epitope, such as IL-5, PDGF-AA, PDGF-BB, TGF beta, TGF  
25 beta2, TGF beta3 and TNF $\alpha$ , for example human TNF Receptor 1 and human TNF $\alpha$ .

30

In a similar aspect, ligands according to the invention can be configured to bind individual epitopes with low affinity, such that binding to individual epitopes is not therapeutically significant; but the increased avidity resulting from binding to two epitopes provides a therapeutic benefit. In a particular example, epitopes may be targeted which are present individually on normal cell types, but present together only on abnormal or diseased cells, such as tumour cells. In such a situation, only the abnormal or

diseased cells are effectively targetted by the bispecific ligands according to the invention.

Ligand specific for multiple copies of the same epitope, or adjacent epitopes, on the same  
5 target (known as chelating dAbs) may also be trimeric or polymeric (tetrameric or more)  
ligands comprising three, four or more non-complementary binding domains. For  
example, ligands may be constructed comprising three or four  $V_H$  domains or  $V_L$   
domains.

10 Moreover, ligands are provided which bind to multisubunit targets, wherein each binding  
domain is specific for a subunit of said target. The ligand may be dimeric, trimeric or  
polymeric.

Preferably, the multi-specific ligands according to the above aspects of the invention are  
15 obtainable by the method of the first aspect of the invention.

According to the above aspect of the second configuration of the invention,  
advantageously the first epitope binding domain and the second epitope binding domains  
are non-complementary immunoglobulin variable domains, as herein defined. That is  
20 either  $V_H$ - $V_H$  or  $V_L$ - $V_L$  variable domains.

Chelating dAbs in particular may be prepared according to a preferred aspect of the  
invention, namely the use of anchor dAbs, in which a library of dimeric, trimeric or  
multimeric dAbs is constructed using a vector which comprises a constant dAb upstream  
25 or downstream of a linker sequence, with a repertoire of second, third and further dAbs  
being inserted on the other side of the linker. For example, the anchor or guiding dAb  
may be TAR1-5 ( $V_K$ ), TAR1-27( $V_K$ ), TAR2h-5( $V_H$ ) or TAR2h-6( $V_K$ ).

In alternative methodologies, the use of linkers may be avoided, for example by the use of  
30 non-covalent bonding or natural affinity between binding domains such as  $V_H$  and  $V_K$ .  
The invention accordingly provides a method for preparing a chelating multimeric ligand  
comprising the steps of:

(a) providing a vector comprising a nucleic acid sequence encoding a single binding domain specific for a first epitope on a target;

(b) providing a vector encoding a repertoire comprising second binding domains specific for a second epitope on said target, which epitope can be the same or different to the first epitope, said second epitope being adjacent to said first epitope; and

(c) expressing said first and second binding domains; and

(d) isolating those combinations of first and second binding domains which combine together to produce a target-binding dimer.

10 The first and second epitopes are adjacent such that a multimeric ligand is capable of binding to both epitopes simultaneously. This provides the ligand with the advantages of increased avidity if binding. Where the epitopes are the same, the increased avidity is obtained by the presence of multiple copies of the epitope on the target, allowing at least two copies to be simultaneously bound in order to obtain the increased avidity effect.

15

The binding domains may be associated by several methods, as well as the use of linkers. For example, the binding domains may comprise cys residues, avidin and streptavidin groups or other means for non-covalent attachment post-synthesis; those combinations which bind to the target efficiently will be isolated. Alternatively, a linker may be present between the first and second binding domains, which are expressed as a single polypeptide from a single vector, which comprises the first binding domain, the linker and a repertoire of second binding domains, for instance as described above.

20

In a preferred aspect, the first and second binding domains associate naturally when bound to antigen; for example,  $V_H$  and  $V_K$  domains, when bound to adjacent epitopes, will naturally associate in a three-way interaction to form a stable dimer. Such associated proteins can be isolated in a target binding assay. An advantage of this procedure is that only binding domains which bind to closely adjacent epitopes, in the correct conformation, will associate and thus be isolated as a result of their increased avidity for the target.

30

In an alternative embodiment of the above aspect of the second configuration of the invention, at least one epitope binding domain comprises a non-immunoglobulin 'protein

scaffold' or 'protein skeleton' as herein defined. Suitable non-immunoglobulin protein scaffolds include but are not limited to any of those selected from the group consisting of: SpA, fibronectin, GroEL and other chaperones, lipocalin, CTLA4 and affibodies, as set forth above.

5

According to the above aspect of the second configuration of the invention, advantageously, the epitope binding domains are attached to a 'protein skeleton'. Advantageously, a protein skeleton according to the invention is an immunoglobulin skeleton.

10

According to the present invention, the term 'immunoglobulin skeleton' refers to a protein which comprises at least one immunoglobulin fold and which acts as a nucleus for one or more epitope binding domains, as defined herein.

15

Preferred immunoglobulin skeletons as herein defined includes any one or more of those selected from the following: an immunoglobulin molecule comprising at least (i) the CL (kappa or lambda subclass) domain of an antibody; or (ii) the CH1 domain of an antibody heavy chain; an immunoglobulin molecule comprising the CH1 and CH2 domains of an antibody heavy chain; an immunoglobulin molecule comprising the CH1, CH2 and CH3 domains of an antibody heavy chain; or any of the subset (ii) in conjunction with the CL (kappa or lambda subclass) domain of an antibody. A hinge region domain may also be included. Such combinations of domains may, for example, mimic natural antibodies, such as IgG or IgM, or fragments thereof, such as Fv, scFv, Fab or F(ab')<sub>2</sub> molecules. Those skilled in the art will be aware that this list is not intended to be exhaustive.

25

Linking of the skeleton to the epitope binding domains, as herein defined may be achieved at the polypeptide level, that is after expression of the nucleic acid encoding the skeleton and/or the epitope binding domains. Alternatively, the linking step may be performed at the nucleic acid level. Methods of linking a protein skeleton according to the present invention, to the one or more epitope binding domains include the use of protein chemistry and/or molecular biology techniques which will be familiar to those skilled in the art and are described herein.

30

Advantageously, the closed conformation multispecific ligand may comprise a first domain capable of binding a target molecule, and a second domain capable of binding a molecule or group which extends the half-life of the ligand. For example, the molecule or group may be a bulky agent, such as HSA or a cell matrix protein. As used herein, the phrase "molecule or group which extends the half-life of a ligand" refers to a molecule or chemical group which, when bound by a dual-specific ligand as described herein increases the in vivo half-life of such dual specific ligand when administered to an animal, relative to a ligand that does not bind that molecule or group. Examples of molecules or groups that extend the half-life of a ligand are described hereinbelow. In a preferred embodiment, the closed conformation multispecific ligand may be capable of binding the target molecule only on displacement of the half-life enhancing molecule or group. Thus, for example, a closed conformation multispecific ligand is maintained in circulation in the bloodstream of a subject by a bulky molecule such as HSA. When a target molecule is encountered, competition between the binding domains of the closed conformation multispecific ligand results in displacement of the HSA and binding of the target.

Ligands according to any aspect of the present invention, as well as dAb monomers useful in constructing such ligands, may advantageously dissociate from their cognate target(s) with a  $K_d$  of 300nM to 5pM (ie,  $3 \times 10^{-7}$  to  $5 \times 10^{-12}$ M), preferably 50nM to 20pM, or 5nM to 200pM or 1nM to 100pM,  $1 \times 10^{-7}$  M or less,  $1 \times 10^{-8}$  M or less,  $1 \times 10^{-9}$  M or less,  $1 \times 10^{-10}$  M or less,  $1 \times 10^{-11}$  M or less; and/or a  $K_{off}$  rate constant of  $5 \times 10^{-1}$  to  $1 \times 10^{-7} \text{ s}^{-1}$ , preferably  $1 \times 10^{-2}$  to  $1 \times 10^{-6} \text{ s}^{-1}$ , or  $5 \times 10^{-3}$  to  $1 \times 10^{-5} \text{ s}^{-1}$ , or  $5 \times 10^{-1} \text{ s}^{-1}$  or less, or  $1 \times 10^{-2} \text{ s}^{-1}$  or less, or  $1 \times 10^{-3} \text{ s}^{-1}$  or less, or  $1 \times 10^{-4} \text{ s}^{-1}$  or less, or  $1 \times 10^{-5} \text{ s}^{-1}$  or less, or  $1 \times 10^{-6} \text{ s}^{-1}$  or less as determined by surface plasmon resonance. The  $K_d$  rate constant is defined as  $K_{off}/K_{on}$ .

In particular the invention provides an anti-TNF $\alpha$  dAb monomer (or dual specific ligand comprising such a dAb), homodimer, heterodimer or homotrimer ligand, wherein each dAb binds TNF $\alpha$ . The ligand binds to TNF $\alpha$  with a  $K_d$  of 300nM to 5pM (ie,  $3 \times 10^{-7}$  to  $5 \times 10^{-12}$ M), preferably 50nM to 20pM, more preferably 5nM to 200pM and most preferably 1nM to 100pM; expressed in an alternative manner, the  $K_d$  is  $1 \times 10^{-7}$  M or less, preferably  $1 \times 10^{-8}$  M or less, more preferably  $1 \times 10^{-9}$  M or less, advantageously  $1 \times$

10<sup>-10</sup> M or less and most preferably 1 x 10<sup>-11</sup> M or less; and/or a K<sub>off</sub> rate constant of 5 x 10<sup>-1</sup> to 1 x 10<sup>-7</sup> S<sup>-1</sup>, preferably 1 x 10<sup>-2</sup> to 1 x 10<sup>-6</sup> S<sup>-1</sup>, more preferably 5 x 10<sup>-3</sup> to 1 x 10<sup>-5</sup> S<sup>-1</sup>, for example 5 x 10<sup>-1</sup> S<sup>-1</sup> or less, preferably 1 x 10<sup>-2</sup> S<sup>-1</sup> or less, more preferably 1 x 10<sup>-3</sup> S<sup>-1</sup> or less, advantageously 1 x 10<sup>-4</sup> S<sup>-1</sup> or less, further advantageously 1 x 10<sup>-5</sup> S<sup>-1</sup> or less,  
5 and most preferably 1 x 10<sup>-6</sup> S<sup>-1</sup> or less, as determined by surface plasmon resonance.

Preferably, the ligand neutralises TNF $\alpha$  in a standard L929 assay with an ND50 of 500nM to 50pM, preferably 100nM to 50pM, advantageously 10nM to 100pM, more preferably 1nM to 100pM; for example 50nM or less, preferably 5nM or less,  
10 advantageously 500pM or less, more preferably 200pM or less and most preferably 100pM or less.

Preferably, the ligand inhibits binding of TNF alpha to TNF alpha Receptor I (p55 receptor) with an IC50 of 500nM to 50pM, preferably 100nM to 50pM, more preferably  
15 10nM to 100pM, advantageously 1nM to 100pM; for example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less. Preferably, the TNF $\alpha$  is Human TNF $\alpha$ .

Furthermore, the invention provides a an anti-TNF Receptor I dAb monomer, or dual  
20 specific ligand comprising such a dAb, that binds to TNF Receptor I with a K<sub>d</sub> of 300nM to 5pM (ie, 3 x 10<sup>-7</sup> to 5 x 10<sup>-12</sup>M), preferably 50nM to 20pM, more preferably 5nM to 200pM and most preferably 1nM to 100pM, for example 1 x 10<sup>-7</sup> M or less, preferably 1 x 10<sup>-8</sup> M or less, more preferably 1 x 10<sup>-9</sup> M or less, advantageously 1 x 10<sup>-10</sup> M or less and most preferably 1 x 10<sup>-11</sup> M or less; and/or a K<sub>off</sub> rate constant of 5 x 10<sup>-1</sup> to 1 x 10<sup>-7</sup>  
25 S<sup>-1</sup>, preferably 1 x 10<sup>-2</sup> to 1 x 10<sup>-6</sup> S<sup>-1</sup>, more preferably 5 x 10<sup>-3</sup> to 1 x 10<sup>-5</sup> S<sup>-1</sup>, for example 5 x 10<sup>-1</sup> S<sup>-1</sup> or less, preferably 1 x 10<sup>-2</sup> S<sup>-1</sup> or less, advantageously 1 x 10<sup>-3</sup> S<sup>-1</sup> or less, more preferably 1 x 10<sup>-4</sup> S<sup>-1</sup> or less, still more preferably 1 x 10<sup>-5</sup> S<sup>-1</sup> or less, and most preferably 1 x 10<sup>-6</sup> S<sup>-1</sup> or less as determined by surface plasmon resonance.

30 Preferably, the dAb monomer or ligand neutralises TNF $\alpha$  in a standard assay (eg, the L929 or HeLa assays described herein) with an ND50 of 500nM to 50pM, preferably 100nM to 50pM, more preferably 10nM to 100pM, advantageously 1nM to 100pM; for

example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less.

Preferably, the dAb monomer or ligand inhibits binding of TNF alpha to TNF alpha Receptor I (p55 receptor) with an IC<sub>50</sub> of 500nM to 50pM, preferably 100nM to 50pM, more preferably 10nM to 100pM, advantageously 1nM to 100pM; for example 50nM or less, preferably 5nM or less, more preferably 500pM or less, advantageously 200pM or less, and most preferably 100pM or less. Preferably, the TNF Receptor I target is Human TNFα.

Furthermore, the invention provides a dAb monomer(or dual specific ligand comprising such a dAb) that binds to serum albumin (SA) with a K<sub>d</sub> of 1nM to 500μM (ie,  $\times 10^{-9}$  to  $5 \times 10^{-4}$ ), preferably 100nM to 10μM. Preferably, for a dual specific ligand comprising a first anti-SA dAb and a second dAb to another target, the affinity (eg K<sub>d</sub> and/or K<sub>off</sub> as measured by surface plasmon resonance, eg using BiaCore) of the second dAb for its target is from 1 to 100000 times (preferably 100 to 100000, more preferably 1000 to 100000, or 10000 to 100000 times) the affinity of the first dAb for SA. For example, the first dAb binds SA with an affinity of approximately 10μM, while the second dAb binds its target with an affinity of 100pM. Preferably, the serum albumin is human serum albumin (HSA).

In one embodiment, the first dAb (or a dAb monomer) binds SA (eg, HSA) with a K<sub>d</sub> of approximately 50, preferably 70, and more preferably 100, 150 or 200 nM.

The invention moreover provides dimers, trimers and polymers of the aforementioned dAb monomers, in accordance with the foregoing aspect of the present invention.

Ligands according to the invention, including dAb monomers, dimers and trimers, can be linked to an antibody Fc region, comprising one or both of C<sub>H</sub>2 and C<sub>H</sub>3 domains, and optionally a hinge region. For example, vectors encoding ligands linked as a single nucleotide sequence to an Fc region may be used to prepare such polypeptides.



In a further aspect of the second configuration of the invention, the present invention provides one or more nucleic acid molecules encoding at least a multispecific ligand as herein defined. In one embodiment, the ligand is a closed conformation ligand. In another embodiment, it is an open conformation ligand. The multispecific ligand may be encoded on a single nucleic acid molecule; alternatively, each epitope binding domain  
5 may be encoded by a separate nucleic acid molecule. Where the ligand is encoded by a single nucleic acid molecule, the domains may be expressed as a fusion polypeptide, or may be separately expressed and subsequently linked together, for example using chemical linking agents. Ligands expressed from separate nucleic acids will be linked  
10 together by appropriate means.

The nucleic acid may further encode a signal sequence for export of the polypeptides from a host cell upon expression and may be fused with a surface component of a filamentous bacteriophage particle (or other component of a selection display system)  
15 upon expression. Leader sequences, which may be used in bacterial expression and/or phage or phagemid display, include pelB, stII, ompA, phoA, bla and pelA.

In a further aspect of the second configuration of the invention the present invention provides a vector comprising nucleic acid according to the present invention.  
20

In a yet further aspect, the present invention provides a host cell transfected with a vector according to the present invention.

Expression from such a vector may be configured to produce, for example on the surface  
25 of a bacteriophage particle, epitope binding domains for selection. This allows selection of displayed domains and thus selection of 'multispecific ligands' using the method of the present invention.

In a preferred embodiment of the second configuration of the invention, the epitope  
30 binding domains are immunoglobulin variable regions and are selected from single domain V gene repertoires. Generally the repertoire of single antibody domains is displayed on the surface of filamentous bacteriophage. In a preferred embodiment each single antibody domain is selected by binding of a phage repertoire to antigen.

The present invention further provides a kit comprising at least a multispecific ligand according to the present invention, which may be an open conformation or closed conformation ligand. Kits according to the invention may be, for example, diagnostic  
5 kits, therapeutic kits, kits for the detection of chemical or biological species, and the like.

In a further aspect still of the second configuration of the invention, the present invention provides a homogenous immunoassay using a ligand according to the present invention.

10 In a further aspect still of the second configuration of the invention, the present invention provides a composition comprising a closed conformation multispecific ligand, obtainable by a method of the present invention, and a pharmaceutically acceptable carrier, diluent or excipient.

15 Moreover, the present invention provides a method for the treatment of disease using a 'closed conformation multispecific ligand' or a composition according to the present invention.

In a preferred embodiment of the invention the disease is cancer or an inflammatory  
20 disease, eg rheumatoid arthritis, asthma or Crohn's disease.

In a further aspect of the second configuration of the invention, the present invention provides a method for the diagnosis, including diagnosis of disease using a closed conformation multispecific ligand, or a composition according to the present invention.  
25 Thus in general the binding of an analyte to a closed conformation multispecific ligand may be exploited to displace an agent, which leads to the generation of a signal on displacement. For example, binding of analyte (second antigen) could displace an enzyme (first antigen) bound to the antibody providing the basis for an immunoassay, especially if the enzyme were held to the antibody through its active site.

30

Thus in a final aspect of the second configuration, the present invention provides a method for detecting the presence of a target molecule, comprising:

- (a) providing a closed conformation multispecific ligand bound to an agent, said ligand being specific for the target molecule and the agent, wherein the agent which is bound by the ligand leads to the generation of a detectable signal on displacement from the ligand;
- (b) exposing the closed conformation multispecific ligand to the target molecule; and
- 5 (c) detecting the signal generated as a result of the displacement of the agent.

According to the above aspect of the second configuration of the invention, advantageously, the agent is an enzyme, which is inactive when bound by the closed conformation multi-specific ligand. Alternatively, the agent may be any one or more  
10 selected from the group consisting of the following: the substrate for an enzyme, and a fluorescent, luminescent or chromogenic molecule which is inactive or quenched when bound by the ligand.

Sequences similar or homologous (e.g., at least about 70% sequence identity) to the  
15 sequences disclosed herein are also part of the invention. In some embodiments, the sequence identity at the amino acid level can be about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. At the nucleic acid level, the sequence identity can be about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,  
20 97%, 98%, 99% or higher. Alternatively, substantial identity exists when the nucleic acid segments will hybridize under selective hybridization conditions (e.g., very high stringency hybridization conditions), to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form.

25 Calculations of "homology" or "sequence identity" or "similarity" between two sequences (the terms are used interchangeably herein) are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes).

30 In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at

corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

Advantageously, the BLAST algorithm (version 2.0) is employed for sequence alignment, with parameters set to default values. The BLAST algorithm is described in detail at the world wide web site ("www") of the National Center for Biotechnology Information ("ncbi") of the National Institutes of Health ("nih") of the U.S. government (".gov"), in the "/Blast/" directory, in the "blast\_help.html" file. The search parameters are defined as follows, and are advantageously set to the defined default parameters.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87(6):2264-8 (see the "blast\_help.html" file, as described above) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. The programs are not generally useful for motif-style searching. For a discussion of basic issues in similarity searching of sequence databases, see Altschul et al. (1994).

The five BLAST programs available at the National Center for Biotechnology Information web site perform the following tasks:

- "blastp" compares an amino acid query sequence against a protein sequence database;
- "blastn" compares a nucleotide query sequence against a nucleotide sequence database;
- "blastx" compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database;
- "tblastn" compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

"tblastx" compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

HISTOGRAM Display a histogram of scores for each search; default is yes. (See  
5 parameter H in the BLAST Manual).

DESCRIPTIONS Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page). See also EXPECT and CUTOFF.

ALIGNMENTS Restricts database sequences to the number specified for which high-  
10 scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

EXPECT The statistical significance threshold for reporting matches against database  
15 sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E  
20 in the BLAST Manual).

CUTOFF Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF  
25 values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

MATRIX Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992, Proc. Natl.  
30 Acad. Sci. USA 89(22):10915-9). The valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

STRAND Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

5 FILTER Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States, 1993, Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see the world wide web site of the NCBI). Filtering can eliminate  
10 statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

15 Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence (e.g., "N" repeated 13 times) and the letter "X" in protein sequences (e.g., "X" repeated 9 times).

Filtering is only applied to the query sequence (or its translation products), not to  
20 database sequences. Default filtering is DUST for BLASTN, SEG for other programs. It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered query sequence  
25 should be suspect.

NCBI-gi Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

30 Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at the NCBI world wide web site described above, in the "/BLAST" directory.

**Brief Description of the Figures**

- Figure 1** shows the diversification of  $V_H$ /HSA at positions H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98 (DVT or NNK encoded respectively) which are in the antigen binding site of  $V_H$  HSA. The sequence of  $V_K$  is diversified at positions L50, L53.
- Figure 2** shows Library 1: Germline  $V_K$ /DVT  $V_H$ ,  
Library 2: Germline  $V_K$ /NNK  $V_H$ ,  
Library 3: Germline  $V_H$ /DVT  $V_K$   
Library 4: Germline  $V_H$ /NNK  $V_K$   
In phage display/ScFv format. These libraries were pre-selected for binding to generic ligands protein A and protein L so that the majority of the clones and selected libraries are functional. Libraries were selected on HSA (first round) and  $\beta$ -gal (second round) or HSA  $\beta$ -gal selection or on  $\beta$ -gal (first round) and HSA (second round)  $\beta$ -gal HSA selection. Soluble scFv from these clones of PCR are amplified in the sequence. One clone encoding a dual specific antibody K8 was chosen for further work.
- Figure 3** shows an alignment of  $V_H$  chains and  $V_K$  chains.
- Figure 4** shows the characterisation of the binding properties of the K8 antibody, the binding properties of the K8 antibody characterised by monoclonal phage ELISA, the dual specific K8 antibody was found to bind HSA and  $\beta$ -gal and displayed on the surface of the phage with absorbant signals greater than 1.0. No cross reactivity with other proteins was detected.
- Figure 5** shows soluble scFv ELISA performed using known concentrations of the K8 antibody fragment. A 96-well plate was coated with 100 $\mu$ g of HSA, BSA and  $\beta$ -gal at 10 $\mu$ g/ml and 100 $\mu$ g/ml of Protein A at 1 $\mu$ g/ml concentration. 50 $\mu$ g of the serial dilutions of the K8 scFv was applied and the bound antibody fragments were detected with Protein L-HRP. ELISA results confirm the dual specific nature of the K8 antibody.

- Figure 6 shows the binding characteristics of the clone K8V<sub>K</sub>/dummy V<sub>H</sub> analysed using soluble scFv ELISA. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al*, Methods Enzymol. 1996;267:83-109 and the supernatant containing scFv assayed directly. Soluble scFv ELISA is performed as described in example 1 and the bound scFvs were detected with Protein L-HRP. The ELISA results revealed that this clone was still able to bind  $\beta$ -gal, whereas binding BSA was abolished.
- 10 Figure 7 shows the sequence of variable domain vectors 1 and 2.
- Figure 8 is a map of the C<sub>H</sub> vector used to construct a V<sub>H</sub>1/V<sub>H</sub>2 multispecific ligand.
- 15 Figure 9 is a map of the V<sub>K</sub> vector used to construct a V<sub>K</sub>1/V<sub>K</sub>2 multispecific ligand.
- Figure 10 TNF receptor assay comparing TAR1-5 dimer 4, TAR1-5-19 dimer 4 and TAR1-5-19 monomer.
- 20 Figure 11 TNF receptor assay comparing TAR1-5 dimers 1-6. All dimers have been FPLC purified and the results for the optimal dimeric species are shown.
- Figure 12 TNF receptor assay of TAR1-5 19 homodimers in different formats: dAb-linker-dAb format with 3U, 5U or 7U linker, Fab format and cysteine hinge linker format.
- 25 Figure 13 Dummy V<sub>H</sub> sequence for library 1. The sequence of the V<sub>H</sub> framework based on germline sequence DP47 – JH4b. Positions where NNK randomisation (N=A or T or C or G nucleotides; K = G or T nucleotides) has been incorporated into library 1 are indicated in bold underlined text.
- 30 Figure 14 Dummy V<sub>H</sub> sequence for library 2. The sequence of the V<sub>H</sub> framework based on germline sequence DP47 – JH4b. Positions where NNK



randomisation (N=A or T or C or G nucleotides; K = G or T nucleotides) has been incorporated into library 2 are indicated in bold underlined text.

5 Figure 15 Dummy  $V_K$  sequence for library 3. The sequence of the  $V_K$  framework based on germline sequence DP $K_9$  - J  $K_1$ . Positions where NNK randomisation (N=A or T or C or G nucleotides; K = G or T nucleotides) has been incorporated into library 3 are indicated in bold underlined text.

10 Figure 16 Nucleotide and amino acid sequence of anti MSA dAbs MSA 16 and MSA 26.

15 Figure 17 Inhibition biacore of MSA 16 and 26. Purified dAbs MSA16 and MSA26 were analysed by inhibition biacore to determine  $K_d$ . Briefly, the dAbs were tested to determine the concentration of dAb required to achieve 200RUs of response on a biacore CM5 chip coated with a high density of MSA. Once the required concentrations of dAb had been determined, MSA antigen at a range of concentrations around the expected  $K_d$  was premixed with the dAb and incubated overnight. Binding to the MSA coated biacore chip of dAb in each of the premixes was then measured at a high flow-rate of 30  $\mu$ l/minute.

20 Figure 18 Serum levels of MSA16 following injection. Serum half life of the dAb MSA16 was determined in mouse. MSA16 was dosed as single i.v. injections at approx 1.5mg/kg into CD1 mice. Modelling with a 2 compartment model showed MSA16 had a  $t_{1/2\alpha}$  of 0.98hr, a  $t_{1/2\beta}$  of 36.5hr and an AUC of 913hr.mg/ml. MSA16 had a considerably lengthened half life compared with HEL4 (an anti-hen egg white lysozyme dAb) which had a  $t_{1/2\alpha}$  of 0.06hr and a  $t_{1/2\beta}$  of 0.34hr.

30 Figure 19 ELISA (a) and TNF receptor assay (c) showing inhibition of TNF binding with a Fab-like fragment comprising MSA26Ck and TAR1-5-19CH. Addition of MSA with the Fab-like fragment reduces the level of inhibition. An ELISA plate coated with 1 $\mu$ g/ml TNF $\alpha$  was probed with

dual specific  $V_{\kappa} C_H$  and  $V_{\kappa} C_{\kappa}$  Fab like fragment and also with a control  $TNF\alpha$  binding dAb at a concentration calculated to give a similar signal on the ELISA. Both the dual specific and control dAb were used to probe the ELISA plate in the presence and in the absence of 2mg/ml MSA. The signal in the dual specific well was reduced by more than 50% but the signal in the dAb well was not reduced at all (see figure 19a). The same dual specific protein was also put into the receptor assay with and without MSA and competition by MSA was also shown (see figure 19c). This demonstrates that binding of MSA to the dual specific is competitive with binding to  $TNF\alpha$ .

**Figure 20**  $TNF$  receptor assay showing inhibition of  $TNF$  binding with a disulphide bonded heterodimer of TAR1-5-19 dAb and MSA16 dAb. Addition of MSA with the dimer reduces the level of inhibition in a dose dependant manner. The  $TNF$  receptor assay (figure 19 (b)) was conducted in the presence of a constant concentration of heterodimer (18nM) and a dilution series of MSA and HSA. The presence of HSA at a range of concentrations (up to 2 mg/ml) did not cause a reduction in the ability of the dimer to inhibit  $TNF\alpha$ . However, the addition of MSA caused a dose dependant reduction in the ability of the dimer to inhibit  $TNF\alpha$  (figure 19a). This demonstrates that MSA and  $TNF\alpha$  compete for binding to the cys bonded TAR1-5-19, MSA16 dimer. MSA and HSA alone did not have an effect on the  $TNF$  binding level in the assay.

## Detailed Description of the Invention

### Definitions

**Complementary** Two immunoglobulin domains are "complementary" where they belong to families of structures which form cognate pairs or groups or are derived from such families and retain this feature. For example, a  $V_H$  domain and a  $V_L$  domain of an antibody are complementary; two  $V_H$  domains are not complementary, and two  $V_L$

domains are not complementary. Complementary domains may be found in other members of the immunoglobulin superfamily, such as the  $V_\alpha$  and  $V_\beta$  (or  $\gamma$  and  $\delta$ ) domains of the T-cell receptor. In the context of the second configuration of the present invention, non-complementary domains do not bind a target molecule cooperatively, but act independently on different target epitopes which may be on the same or different molecules. Domains which are artificial, such as domains based on protein scaffolds which do not bind epitopes unless engineered to do so, are non-complementary. Likewise, two domains based on (for example) an immunoglobulin domain and a fibronectin domain are not complementary.

**Immunoglobulin** This refers to a family of polypeptides which retain the immunoglobulin fold characteristic of antibody molecules, which contains two  $\beta$  sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions *in vivo*, including widespread roles in the immune system (for example, antibodies, T-cell receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules) and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily molecules which possess binding domains. Preferably, the present invention relates to antibodies.

**Combining** Variable domains according to the invention are combined to form a group of domains; for example, complementary domains may be combined, such as  $V_L$  domains being combined with  $V_H$  domains. Non-complementary domains may also be combined. Domains may be combined in a number of ways, involving linkage of the domains by covalent or non-covalent means.

**Domain** A domain is a folded protein structure which retains its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain. By single antibody variable domain is meant a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes complete antibody variable domains and modified variable domains, for example

in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains which retain at least in part the binding activity and specificity of the full-length domain.

5

**Repertoire** A collection of diverse variants, for example polypeptide variants which differ in their primary sequence. A library used in the present invention will encompass a repertoire of polypeptides comprising at least 1000 members.

10 **Library** The term library refers to a mixture of heterogeneous polypeptides or nucleic acids. The library is composed of members, each of which have a single polypeptide or nucleic acid sequence. To this extent, *library* is synonymous with *repertoire*. Sequence differences between library members are responsible for the diversity present in the library. The library may take the form of a simple mixture of  
15 polypeptides or nucleic acids, or may be in the form of organisms or cells, for example bacteria, viruses, animal or plant cells and the like, transformed with a library of nucleic acids. Preferably, each individual organism or cell contains only one or a limited number of library members. Advantageously, the nucleic acids are incorporated into expression vectors, in order to allow expression of the polypeptides encoded by the nucleic acids. In  
20 a preferred aspect, therefore, a library may take the form of a population of host organisms, each organism containing one or more copies of an expression vector containing a single member of the library in nucleic acid form which can be expressed to produce its corresponding polypeptide member. Thus, the population of host organisms has the potential to encode a large repertoire of genetically diverse polypeptide variants.

25

A 'closed conformation multi-specific ligand' describes a multi-specific ligand as herein defined comprising at least two epitope binding domains as herein defined. The term 'closed conformation' (multi-specific ligand) means that the epitope binding domains of the ligand are arranged such that epitope binding by one epitope binding  
30 domain competes with epitope binding by another epitope binding domain. That is, cognate epitopes may be bound by each epitope binding domain individually but not simultaneously. The closed conformation of the ligand can be achieved using methods herein described.

**Antibody** An antibody (for example IgG, IgM, IgA, IgD or IgE) or fragment (such as a Fab, F(ab')<sub>2</sub>, Fv, disulphide linked Fv, scFv, closed conformation multispecific antibody, disulphide-linked scFv, diabody) whether derived from any species naturally producing an antibody, or created by recombinant DNA technology; whether isolated  
5 from serum, B-cells, hybridomas, transfectomas, yeast or bacteria).

**Dual-specific ligand** A ligand comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain as herein defined, wherein the variable regions are capable of binding to two different antigens or two epitopes on the  
10 same antigen which are not normally bound by a monospecific immunoglobulin. For example, the two epitopes may be on the same hapten, but are not the same epitope or sufficiently adjacent to be bound by a monospecific ligand. The dual specific ligands according to the invention are composed of variable domains which have different specificities, and do not contain mutually complementary variable domain pairs which  
15 have the same specificity.

**Antigen** A molecule that is bound by a ligand according to the present invention. Typically, antigens are bound by antibody ligands and are capable of raising an antibody response *in vivo*. It may be a polypeptide, protein, nucleic acid or other molecule.  
20 Generally, the dual specific ligands according to the invention are selected for target specificity against a particular antigen. In the case of conventional antibodies and fragments thereof, the antibody binding site defined by the variable loops (L1, L2, L3 and H1, H2, H3) is capable of binding to the antigen.

**Epitope** A unit of structure conventionally bound by an immunoglobulin V<sub>H</sub>/V<sub>L</sub> pair. Epitopes define the minimum binding site for an antibody, and thus represent the target of specificity of an antibody. In the case of a single domain antibody, an epitope represents the unit of structure bound by a variable domain in isolation.  
25

**Generic ligand** A ligand that binds to all members of a repertoire. Generally, not bound through the antigen binding site as defined above. Non-limiting examples include protein A, protein L and protein G.  
30

**Selecting** Derived by screening, or derived by a Darwinian selection process, in which binding interactions are made between a domain and the antigen or epitope or between an antibody and an antigen or epitope. Thus a first variable domain may be selected for binding to an antigen or epitope in the presence or in the absence of a complementary variable domain.

**Universal framework** A single antibody framework sequence corresponding to the regions of an antibody conserved in sequence as defined by Kabat ("Sequences of Proteins of Immunological Interest", US Department of Health and Human Services) or corresponding to the human germline immunoglobulin repertoire or structure as defined by Chothia and Lesk, (1987) J. Mol. Biol. 196:910-917. The invention provides for the use of a single framework, or a set of such frameworks, which has been found to permit the derivation of virtually any binding specificity through variation in the hypervariable regions alone.

**Half-life** The time taken for the serum concentration of the ligand to reduce by 50%, *in vivo*, for example due to degradation of the ligand and/or clearance or sequestration of the ligand by natural mechanisms. The ligands of the invention are stabilised *in vivo* and their half-life increased by binding to molecules which resist degradation and/or clearance or sequestration. Typically, such molecules are naturally occurring proteins which themselves have a long half-life *in vivo*. The half-life of a ligand is increased if its functional activity persists, *in vivo*, for a longer period than a similar ligand which is not specific for the half-life increasing molecule. Thus, a ligand specific for HSA and a target molecule is compared with the same ligand wherein the specificity for HSA is not present, that it does not bind HSA but binds another molecule. For example, it may bind a second epitope on the target molecule. Typically, the half life is increased by 10%, 20%, 30%, 40%, 50% or more. Increases in the range of 2x, 3x, 4x, 5x, 10x, 20x, 30x, 40x, 50x or more of the half life are possible. Alternatively, or in addition, increases in the range of up to 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 150x of the half life are possible.

**Homogeneous immunoassay** An immunoassay in which analyte is detected without need for a step of separating bound and un-bound reagents.

**Substantially identical (or "substantially homologous")**

A first amino acid or nucleotide sequence that contains a sufficient number of identical or equivalent (*e.g.*, with a similar side chain, *e.g.*, conserved amino acid substitutions) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have similar activities. In the case of antibodies, the second antibody has the same binding specificity and has at least 50% of the affinity of the same.

As used herein, the terms "low stringency," "medium stringency," "high stringency," or "very high stringency conditions" describe conditions for nucleic acid hybridization and washing. Guidance for performing hybridization reactions can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6, which is incorporated herein by reference in its entirety. Aqueous and nonaqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: (1) low stringency hybridization conditions in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50°C (the temperature of the washes can be increased to 55°C for low stringency conditions); (2) medium stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C; (3) high stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and preferably (4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C. Very high stringency conditions (4) are the preferred conditions and the ones that should be used unless otherwise specified.

**Detailed Description of the Invention**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (*e.g.*, in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see

generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4<sup>th</sup> Ed, John Wiley & Sons, Inc. which are incorporated herein by reference) and chemical methods.

5

### Preparation of immunoglobulin based multi-specific ligands

Dual specific ligands according to the invention, whether open or closed in conformation according to the desired configuration of the invention, may be prepared according to  
10 previously established techniques, used in the field of antibody engineering, for the preparation of scFv, "phage" antibodies and other engineered antibody molecules. Techniques for the preparation of antibodies, and in particular bispecific antibodies, are for example described in the following reviews and the references cited therein: Winter & Milstein, (1991) Nature 349:293-299; Plueckthun (1992) Immunological Reviews  
15 130:151-188; Wright *et al.*, (1992) Crit. Rev. Immunol. 12:125-168; Holliger, P. & Winter, G. (1993) Curr. Op. Biotechn. 4, 446-449; Carter, *et al.* (1995) J. Hematother. 4, 463-470; Chester, K.A. & Hawkins, R.E. (1995) Trends Biotechn. 13, 294-300; Hoogenboom, H.R. (1997) Nature Biotechnol. 15, 125-126; Fearon, D. (1997) Nature Biotechnol. 15, 618-619; Plückthun, A. & Pack, P. (1997) Immunotechnology 3, 83-105;  
20 Carter, P. & Merchant, A.M. (1997) Curr. Opin. Biotechnol. 8, 449-454; Holliger, P. & Winter, G. (1997) Cancer Immunol. Immunother. 45, 128-130.

The invention provides for the selection of variable domains against two different antigens or epitopes, and subsequent combination of the variable domains.

25

The techniques employed for selection of the variable domains employ libraries and selection procedures which are known in the art. Natural libraries (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581; Vaughan *et al.* (1996) *Nature Biotech.*, 14: 309) which use rearranged V genes harvested from human B cells are well known to those skilled in the  
30 art. Synthetic libraries (Hoogenboom & Winter (1992) *J. Mol. Biol.*, 227: 381; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89: 4457; Nissim *et al.* (1994) *EMBO J.*, 13: 692; Griffiths *et al.* (1994) *EMBO J.*, 13: 3245; De Kruif *et al.* (1995) *J. Mol. Biol.*, 248: 97) are prepared by cloning immunoglobulin V genes, usually using PCR. Errors in the PCR



process can lead to a high degree of randomisation.  $V_H$  and/or  $V_L$  libraries may be selected against target antigens or epitopes separately, in which case single domain binding is directly selected for, or together.

- 5 A preferred method for making a dual specific ligand according to the present invention comprises using a selection system in which a repertoire of variable domains is selected for binding to a first antigen or epitope and a repertoire of variable domains is selected for binding to a second antigen or epitope. The selected variable first and second variable domains are then combined and the dual-specific ligand selected for binding to both first  
10 and second antigen or epitope. Closed conformation ligands are selected for binding both first and second antigen or epitope in isolation but not simultaneously.

A. Library vector systems

- 15 A variety of selection systems are known in the art which are suitable for use in the present invention. Examples of such systems are described below.

Bacteriophage lambda expression systems may be screened directly as bacteriophage plaques or as colonies of lysogens, both as previously described (Huse *et al.* (1989)  
20 *Science*, 246: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87; Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87: 8095; Persson *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, 88: 2432) and are of use in the invention. Whilst such expression systems can be used to screen up to  $10^6$  different members of a library, they are not really suited to screening of larger numbers (greater than  $10^6$  members).

- 25 Of particular use in the construction of libraries are selection display systems, which enable a nucleic acid to be linked to the polypeptide it expresses. As used herein, a selection display system is a system that permits the selection, by suitable display means, of the individual members of the library by binding the generic and/or target ligands.

30 Selection protocols for isolating desired members of large libraries are known in the art, as typified by phage display techniques. Such systems, in which diverse peptide sequences are displayed on the surface of filamentous bacteriophage (Scott and Smith

(1990) *Science*, 249: 386), have proven useful for creating libraries of antibody fragments (and the nucleotide sequences that encoding them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen (McCafferty *et al.*, WO 92/01047). The nucleotide sequences encoding the V<sub>H</sub> and V<sub>L</sub> regions are linked to  
5 gene fragments which encode leader signals that direct them to the periplasmic space of *E. coli* and as a result the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids (phagebodies). An advantage of phage-based display systems is that, because they are  
10 biological systems, selected library members can be amplified simply by growing the phage containing the selected library member in bacterial cells. Furthermore, since the nucleotide sequence that encode the polypeptide library member is contained on a phage or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

15 Methods for the construction of bacteriophage antibody display libraries and lambda phage expression libraries are well known in the art (McCafferty *et al.* (1990) *Nature*, 348: 552; Kang *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, 88: 4363; Clackson *et al.* (1991) *Nature*, 352: 624; Lowman *et al.* (1991) *Biochemistry*, 30: 10832; Burton *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, 88: 10134; Hoogenboom *et al.* (1991) *Nucleic Acids Res.*, 19: 4133; Chang *et al.* (1991) *J. Immunol.*, 147: 3610; Breitling *et al.* (1991) *Gene*, 104: 147; Marks *et al.* (1991) *supra*; Barbas *et al.* (1992) *supra*; Hawkins and Winter (1992) *J. Immunol.*, 22: 867; Marks *et al.*, 1992, *J. Biol. Chem.*, 267: 16007; Lerner *et al.* (1992) *Science*, 258: 1313, incorporated herein by reference).

25 One particularly advantageous approach has been the use of scFv phage-libraries (Huston *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.*, 85: 5879-5883; Chaudhary *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87: 1066-1070; McCafferty *et al.* (1990) *supra*; Clackson *et al.* (1991) *Nature*, 352: 624; Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581; Chiswell *et al.* (1992) *Trends Biotech.*, 10: 80; Marks *et al.* (1992) *J. Biol. Chem.*, 267). Various  
30 embodiments of scFv libraries displayed on bacteriophage coat proteins have been described. Refinements of phage display approaches are also known, for example as

described in WO96/06213 and WO92/01047 (Medical Research Council *et al.*) and WO97/08320 (Morphosys), which are incorporated herein by reference.

Other systems for generating libraries of polypeptides involve the use of cell-free enzymatic machinery for the *in vitro* synthesis of the library members. In one method, RNA molecules are selected by alternate rounds of selection against a target ligand and PCR amplification (Tuerk and Gold (1990) *Science*, 249: 505; Ellington and Szostak (1990) *Nature*, 346: 818). A similar technique may be used to identify DNA sequences which bind a predetermined human transcription factor (Thiesen and Bach (1990) *Nucleic Acids Res.*, 18: 3203; Beaudry and Joyce (1992) *Science*, 257: 635; WO92/05258 and WO92/14843). In a similar way, *in vitro* translation can be used to synthesise polypeptides as a method for generating large libraries. These methods which generally comprise stabilised polysome complexes, are described further in WO88/08453, WO90/05785, WO90/07003, WO91/02076, WO91/05058, and WO92/02536. Alternative display systems which are not phage-based, such as those disclosed in WO95/22625 and WO95/11922 (Affymax) use the polysomes to display polypeptides for selection.

A still further category of techniques involves the selection of repertoires in artificial compartments, which allow the linkage of a gene with its gene product. For example, a selection system in which nucleic acids encoding desirable gene products may be selected in microcapsules formed by water-in-oil emulsions is described in WO99/02671, WO00/40712 and Tawfik & Griffiths (1998) *Nature Biotechnol* 16(7), 652-6. Genetic elements encoding a gene product having a desired activity are compartmentalised into microcapsules and then transcribed and/or translated to produce their respective gene products (RNA or protein) within the microcapsules. Genetic elements which produce gene product having desired activity are subsequently sorted. This approach selects gene products of interest by detecting the desired activity by a variety of means.

#### 30 B. Library Construction.

Libraries intended for selection, may be constructed using techniques known in the art, for example as set forth above, or may be purchased from commercial sources. Libraries

which are useful in the present invention are described, for example, in WO99/20749. Once a vector system is chosen and one or more nucleic acid sequences encoding polypeptides of interest are cloned into the library vector, one may generate diversity within the cloned molecules by undertaking mutagenesis prior to expression; alternatively, the encoded proteins may be expressed and selected, as described above, before mutagenesis and additional rounds of selection are performed. Mutagenesis of nucleic acid sequences encoding structurally optimised polypeptides is carried out by standard molecular methods. Of particular use is the polymerase chain reaction, or PCR, (Mullis and Faloona (1987) *Methods Enzymol.*, 155: 335, herein incorporated by reference). PCR, which uses multiple cycles of DNA replication catalysed by a thermostable, DNA-dependent DNA polymerase to amplify the target sequence of interest, is well known in the art. The construction of various antibody libraries has been discussed in Winter *et al.* (1994) *Ann. Rev. Immunology* 12, 433-55, and references cited therein.

PCR is performed using template DNA (at least 1fg; more usefully, 1-1000 ng) and at least 25 pmol of oligonucleotide primers; it may be advantageous to use a larger amount of primer when the primer pool is heavily heterogeneous, as each sequence is represented by only a small fraction of the molecules of the pool, and amounts become limiting in later amplification cycles. A typical reaction mixture includes: 2µl of DNA, 25 pmol of oligonucleotide primer, 2.5 µl of 10X PCR buffer 1 (Perkin-Elmer, Foster City, CA), 0.4 µl of 1.25 µM dNTP, 0.15 µl (or 2.5 units) of Taq DNA polymerase (Perkin Elmer, Foster City, CA) and deionized water to a total volume of 25 µl. Mineral oil is overlaid and the PCR is performed using a programmable thermal cycler. The length and temperature of each step of a PCR cycle, as well as the number of cycles, is adjusted in accordance to the stringency requirements in effect. Annealing temperature and timing are determined both by the efficiency with which a primer is expected to anneal to a template and the degree of mismatch that is to be tolerated; obviously, when nucleic acid molecules are simultaneously amplified and mutagenised, mismatch is required, at least in the first round of synthesis. The ability to optimise the stringency of primer annealing conditions is well within the knowledge of one of moderate skill in the art. An annealing temperature of between 30 °C and 72 °C is used. Initial denaturation of the template molecules normally occurs at between 92°C and 99°C for 4 minutes, followed by 20-40

cycles consisting of denaturation (94-99°C for 15 seconds to 1 minute), annealing (temperature determined as discussed above; 1-2 minutes), and extension (72°C for 1-5 minutes, depending on the length of the amplified product). Final extension is generally for 4 minutes at 72°C, and may be followed by an indefinite (0-24 hour) step at 4°C.

5

C. Combining single variable domains

Domains useful in the invention, once selected, may be combined by a variety of methods known in the art, including covalent and non-covalent methods.

10

Preferred methods include the use of polypeptide linkers, as described, for example, in connection with scFv molecules (Bird *et al.*, (1988) Science 242:423-426). Discussion of suitable linkers is provided in Bird *et al.* Science 242, 423-426; Hudson *et al.*, Journal Immunol Methods 231 (1999) 177-189; Hudson *et al.*, Proc Nat Acad Sci USA 85, 5879-5883. Linkers are preferably flexible, allowing the two single domains to interact. One linker example is a (Gly<sub>4</sub> Ser)<sub>n</sub> linker, where n=1 to 8, eg, 2, 3, 4, 5 or 7. The linkers used in diabodies, which are less flexible, may also be employed (Holliger *et al.*, (1993) PNAS (USA) 90:6444-6448).

15

20 In one embodiment, the linker employed is not an immunoglobulin hinge region.

Variable domains may be combined using methods other than linkers. For example, the use of disulphide bridges, provided through naturally-occurring or engineered cysteine residues, may be exploited to stabilise V<sub>H</sub>-V<sub>H</sub>, V<sub>L</sub>-V<sub>L</sub> or V<sub>H</sub>-V<sub>L</sub> dimers (Reiter *et al.*, (1994) Protein Eng. 7:697-704) or by remodelling the interface between the variable domains to improve the "fit" and thus the stability of interaction (Ridgeway *et al.*, (1996) Protein Eng. 7:617-621; Zhu *et al.*, (1997) Protein Science 6:781-788).

25

Other techniques for joining or stabilising variable domains of immunoglobulins, and in particular antibody V<sub>H</sub> domains, may be employed as appropriate.

30

In accordance with the present invention, dual specific ligands can be in "closed" conformations in solution. A "closed" configuration is that in which the two domains (for

example  $V_H$  and  $V_L$ ) are present in associated form, such as that of an associated  $V_H$ - $V_L$  pair which forms an antibody binding site. For example, scFv may be in a closed conformation, depending on the arrangement of the linker used to link the  $V_H$  and  $V_L$  domains. If this is sufficiently flexible to allow the domains to associate, or rigidly holds  
5 them in the associated position, it is likely that the domains will adopt a closed conformation.

Similarly,  $V_H$  domain pairs and  $V_L$  domain pairs may exist in a closed conformation. Generally, this will be a function of close association of the domains, such as by a rigid  
10 linker, in the ligand molecule. Ligands in a closed conformation will be unable to bind both the molecule which increases the half-life of the ligand and a second target molecule. Thus, the ligand will typically only bind the second target molecule on dissociation from the molecule which increases the half-life of the ligand.

15 Moreover, the construction of  $V_H/V_H$ ,  $V_L/V_L$  or  $V_H/V_L$  dimers without linkers provides for competition between the domains.

Ligands according to the invention may moreover be in an open conformation. In such a conformation, the ligands will be able to simultaneously bind both the molecule which  
20 increases the half-life of the ligand and the second target molecule. Typically, variable domains in an open configuration are (in the case of  $V_H$ - $V_L$  pairs) held far enough apart for the domains not to interact and form an antibody binding site and not to compete for binding to their respective epitopes. In the case of  $V_H/V_H$  or  $V_L/V_L$  dimers, the domains are not forced together by rigid linkers. Naturally, such domain pairings will not compete  
25 for antigen binding or form an antibody binding site.

Fab fragments and whole antibodies will exist primarily in the closed conformation, although it will be appreciated that open and closed dual specific ligands are likely to exist in a variety of equilibria under different circumstances. Binding of the ligand to a  
30 target is likely to shift the balance of the equilibrium towards the open configuration. Thus, certain ligands according to the invention can exist in two conformations in solution, one of which (the open form) can bind two antigens or epitopes independently,

whilst the alternative conformation (the closed form) can only bind one antigen or epitope; antigens or epitopes thus compete for binding to the ligand in this conformation.

Although the open form of the dual specific ligand may thus exist in equilibrium with the closed form in solution, it is envisaged that the equilibrium will favour the closed form; moreover, the open form can be sequestered by target binding into a closed conformation. Preferably, therefore, certain dual specific ligands of the invention are present in an equilibrium between two (open and closed) conformations.

Dual specific ligands according to the invention may be modified in order to favour an open or closed conformation. For example, stabilisation of  $V_H$ - $V_L$  interactions with disulphide bonds stabilises the closed conformation. Moreover, linkers used to join the domains, including  $V_H$  domain and  $V_L$  domain pairs, may be constructed such that the open form is favoured; for example, the linkers may sterically hinder the association of the domains, such as by incorporation of large amino acid residues in opportune locations, or the designing of a suitable rigid structure which will keep the domains physically spaced apart.

#### D. Characterisation of the dual-specific ligand.

20

The binding of the dual-specific ligand to its specific antigens or epitopes can be tested by methods which will be familiar to those skilled in the art and include ELISA. In a preferred embodiment of the invention binding is tested using monoclonal phage ELISA.

Phage ELISA may be performed according to any suitable procedure: an exemplary protocol is set forth below.

Populations of phage produced at each round of selection can be screened for binding by ELISA to the selected antigen or epitope, to identify "polyclonal" phage antibodies. Phage from single infected bacterial colonies from these populations can then be screened by ELISA to identify "monoclonal" phage antibodies. It is also desirable to screen soluble antibody fragments for binding to antigen or epitope, and this can also be undertaken by

ELISA using reagents, for example, against a C- or N-terminal tag (see for example Winter *et al.* (1994) *Ann. Rev. Immunology* 12, 433-55 and references cited therein).

The diversity of the selected phage monoclonal antibodies may also be assessed by gel electrophoresis of PCR products (Marks *et al.* 1991, *supra*; Nissim *et al.* 1994 *supra*), probing (Tomlinson *et al.*, 1992) *J. Mol. Biol.* 227, 776) or by sequencing of the vector DNA.

E. Structure of 'Dual-specific ligands'.

10

As described above, an antibody is herein defined as an antibody (for example IgG, IgM, IgA, IgA, IgE) or fragment (Fab, Fv, disulphide linked Fv, scFv, diabody) which comprises at least one heavy and a light chain variable domain, at least two heavy chain variable domains or at least two light chain variable domains. It may be at least partly derived from any species naturally producing an antibody, or created by recombinant DNA technology; whether isolated from serum, B-cells, hybridomas, transfectomas, yeast or bacteria).

20

In a preferred embodiment of the invention the dual-specific ligand comprises at least one single heavy chain variable domain of an antibody and one single light chain variable domain of an antibody, or two single heavy or light chain variable domains. For example, the ligand may comprise a  $V_H/V_L$  pair, a pair of  $V_H$  domains or a pair of  $V_L$  domains.

25

The first and the second variable domains of such a ligand may be on the same polypeptide chain. Alternatively they may be on separate polypeptide chains. In the case that they are on the same polypeptide chain they may be linked by a linker, which is preferentially a peptide sequence, as described above.

30

The first and second variable domains may be covalently or non-covalently associated. In the case that they are covalently associated, the covalent bonds may be disulphide bonds.

In the case that the variable domains are selected from V-gene repertoires selected for instance using phage display technology as herein described, then these variable domains



comprise a universal framework region, such that is they may be recognised by a specific generic ligand as herein defined. The use of universal frameworks, generic ligands and the like is described in WO99/20749.

5 Where V-gene repertoires are used variation in polypeptide sequence is preferably located within the structural loops of the variable domains. The polypeptide sequences of either variable domain may be altered by DNA shuffling or by mutation in order to enhance the interaction of each variable domain with its complementary pair. DNA shuffling is known in the art and taught, for example, by Stemmer, 1994, Nature 370: 389-391 and  
10 U.S. Patent No. 6,297,053, both of which are incorporated herein by reference. Other methods of mutagenesis are well known to those of skill in the art.

In a preferred embodiment of the invention the 'dual-specific ligand' is a single chain Fv fragment. In an alternative embodiment of the invention, the 'dual-specific ligand'  
15 consists of a Fab format.

In a further aspect, the present invention provides nucleic acid encoding at least a 'dual-specific ligand' as herein defined.

20 One skilled in the art will appreciate that, depending on the aspect of the invention, both antigens or epitopes may bind simultaneously to the same antibody molecule. Alternatively, they may compete for binding to the same antibody molecule. For example, where both epitopes are bound simultaneously, both variable domains of a dual specific ligand are able to independently bind their target epitopes. Where the domains  
25 compete, the one variable domain is capable of binding its target, but not at the same time as the other variable domain binds its cognate target; or the first variable domain is capable of binding its target, but not at the same time as the second variable domain binds its cognate target.

30 The variable regions may be derived from antibodies directed against target antigens or epitopes. Alternatively they may be derived from a repertoire of single antibody domains such as those expressed on the surface of filamentous bacteriophage. Selection may be performed as described below.

In general, the nucleic acid molecules and vector constructs required for the performance of the present invention may be constructed and manipulated as set forth in standard laboratory manuals, such as Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory*  
5 *Manual*, Cold Spring Harbor, USA.

The manipulation of nucleic acids useful in the present invention is typically carried out in recombinant vectors.

10 Thus in a further aspect, the present invention provides a vector comprising nucleic acid encoding at least a 'dual-specific ligand' as herein defined.

As used herein, vector refers to a discrete element that is used to introduce heterologous DNA into cells for the expression and/or replication thereof. Methods by which to select  
15 or construct and, subsequently, use such vectors are well known to one of ordinary skill in the art. Numerous vectors are publicly available, including bacterial plasmids, bacteriophage, artificial chromosomes and episomal vectors. Such vectors may be used for simple cloning and mutagenesis; alternatively gene expression vector is employed. A vector of use according to the invention may be selected to accommodate a polypeptide  
20 coding sequence of a desired size, typically from 0.25 kilobase (kb) to 40 kb or more in length. A suitable host cell is transformed with the vector after *in vitro* cloning manipulations. Each vector contains various functional components, which generally include a cloning (or "polylinker") site, an origin of replication and at least one selectable marker gene. If given vector is an expression vector, it additionally possesses one or more  
25 of the following: enhancer element, promoter, transcription termination and signal sequences, each positioned in the vicinity of the cloning site, such that they are operatively linked to the gene encoding a ligand according to the invention.

Both cloning and expression vectors generally contain nucleic acid sequences that enable  
30 the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses.

The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors unless these are  
5 used in mammalian cells able to replicate high levels of DNA, such as COS cells.

Advantageously, a cloning or expression vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not  
10 transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

15 Since the replication of vectors encoding a ligand according to the present invention is most conveniently performed in *E. coli*, an *E. coli*-selectable marker, for example, the  $\beta$ -lactamase gene that confers resistance to the antibiotic ampicillin, is of use. These can be obtained from *E. coli* plasmids, such as pBR322 or a pUC plasmid such as pUC18 or  
20 pUC19.

Expression vectors usually contain a promoter that is recognised by the host organism and is operably linked to the coding sequence of interest. Such a promoter may be inducible or constitutive. The term "operably linked" refers to a juxtaposition wherein the  
25 components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

30 Promoters suitable for use with prokaryotic hosts include, for example, the  $\beta$ -lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Promoters for use in bacterial systems

will also generally contain a Shine-Delgarno sequence operably linked to the coding sequence.

The preferred vectors are expression vectors that enables the expression of a nucleotide  
5 sequence corresponding to a polypeptide library member. Thus, selection with the first  
and/or second antigen or epitope can be performed by separate propagation and  
expression of a single clone expressing the polypeptide library member or by use of any  
selection display system. As described above, the preferred selection display system is  
bacteriophage display. Thus, phage or phagemid vectors may be used, eg pIT1 or pIT2.  
10 Leader sequences useful in the invention include pelB, stII, ompA, phoA, bla and pelA.  
One example are phagemid vectors which have an *E. coli*. origin of replication (for  
double stranded replication) and also a phage origin of replication (for production of  
single-stranded DNA). The manipulation and expression of such vectors is well known in  
the art (Hoogenboom and Winter (1992) supra; Nissim *et al.* (1994) supra). Briefly, the  
15 vector contains a  $\beta$ -lactamase gene to confer selectivity on the phagemid and a lac  
promoter upstream of a expression cassette that consists (N to C terminal) of a pelB  
leader sequence (which directs the expressed polypeptide to the periplasmic space), a  
multiple cloning site (for cloning the nucleotide version of the library member),  
optionally, one or more peptide tag (for detection), optionally, one or more TAG stop  
20 codon and the phage protein pIII. Thus, using various suppressor and non-suppressor  
strains of *E. coli* and with the addition of glucose, iso-propyl thio- $\beta$ -D-galactoside (IPTG)  
or a helper phage, such as VCS M13, the vector is able to replicate as a plasmid with no  
expression, produce large quantities of the polypeptide library member only or produce  
phage, some of which contain at least one copy of the polypeptide-pIII fusion on their  
25 surface.

Construction of vectors encoding ligands according to the invention employs  
conventional ligation techniques. Isolated vectors or DNA fragments are cleaved,  
tailored, and religated in the form desired to generate the required vector. If desired,  
30 analysis to confirm that the correct sequences are present in the constructed vector can be  
performed in a known fashion. Suitable methods for constructing expression vectors,  
preparing *in vitro* transcripts, introducing DNA into host cells, and performing analyses  
for assessing expression and function are known to those skilled in the art. The presence

of a gene sequence in a sample is detected, or its amplification and/or expression quantified by conventional methods, such as Southern or Northern analysis, Western blotting, dot blotting of DNA, RNA or protein, *in situ* hybridisation, immunocytochemistry or sequence analysis of nucleic acid or protein molecules. Those skilled in the art will readily envisage how these methods may be modified, if desired.

### Structure of closed conformation multispecific ligands

According to one aspect of the second configuration of the invention present invention, the two or more non-complementary epitope binding domains are linked so that they are in a closed conformation as herein defined. Advantageously, they may be further attached to a skeleton which may, as a alternative, or on addition to a linker described herein, facilitate the formation and/or maintenance of the closed conformation of the epitope binding sites with respect to one another.

#### (I) Skeletons

Skeletons may be based on immunoglobulin molecules or may be non-immunoglobulin in origin as set forth above. Preferred immunoglobulin skeletons as herein defined includes any one or more of those selected from the following: an immunoglobulin molecule comprising at least (i) the CL (kappa or lambda subclass) domain of an antibody; or (ii) the CH1 domain of an antibody heavy chain; an immunoglobulin molecule comprising the CH1 and CH2 domains of an antibody heavy chain; an immunoglobulin molecule comprising the CH1, CH2 and CH3 domains of an antibody heavy chain; or any of the subset (ii) in conjunction with the CL (kappa or lambda subclass) domain of an antibody. A hinge region domain may also be included.. Such combinations of domains may, for example, mimic natural antibodies, such as IgG or IgM, or fragments thereof, such as Fv, scFv, Fab or F(ab')<sub>2</sub> molecules. Those skilled in the art will be aware that this list is not intended to be exhaustive.

#### (II) Protein scaffolds

Each epitope binding domain comprises a protein scaffold and one or more CDRs which are involved in the specific interaction of the domain with one or more epitopes. Advantageously, an epitope binding domain according to the present invention comprises

three CDRs. Suitable protein scaffolds include any of those selected from the group consisting of the following: those based on immunoglobulin domains, those based on fibronectin, those based on affibodies, those based on CTLA4, those based on chaperones such as GroEL, those based on lipocalin and those based on the bacterial Fc receptors SpA and SpD. Those skilled in the art will appreciate that this list is not intended to be exhaustive.

F: Scaffolds for use in Constructing Dual Specific Ligands

10 i. Selection of the main-chain conformation

The members of the immunoglobulin superfamily all share a similar fold for their polypeptide chain. For example, although antibodies are highly diverse in terms of their primary sequence, comparison of sequences and crystallographic structures has revealed that, contrary to expectation, five of the six antigen binding loops of antibodies (H1, H2, L1, L2, L3) adopt a limited number of main-chain conformations, or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, 196: 901; Chothia *et al.* (1989) *Nature*, 342: 877). Analysis of loop lengths and key residues has therefore enabled prediction of the main-chain conformations of H1, H2, L1, L2 and L3 found in the majority of human antibodies (Chothia *et al.* (1992) *J. Mol. Biol.*, 227: 799; Tomlinson *et al.* (1995) *EMBO J.*, 14: 4628; Williams *et al.* (1996) *J. Mol. Biol.*, 264: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, 263: 800; Shirai *et al.* (1996) *FEBS Letters*, 399: 1).

The dual specific ligands of the present invention are advantageously assembled from libraries of domains, such as libraries of V<sub>H</sub> domains and/or libraries of V<sub>L</sub> domains. Moreover, the dual specific ligands of the invention may themselves be provided in the form of libraries. In one aspect of the present invention, libraries of dual specific ligands and/or domains are designed in which certain loop lengths and key residues have been chosen to ensure that the main-chain conformation of the members is known. Advantageously, these are real conformations of immunoglobulin superfamily molecules

found in nature, to minimise the chances that they are non-functional, as discussed above. Germline V gene segments serve as one suitable basic framework for constructing antibody or T-cell receptor libraries; other sequences are also of use. Variations may occur at a low frequency, such that a small number of functional members may possess an altered main-chain conformation, which does not affect its function.

Canonical structure theory is also of use to assess the number of different main-chain conformations encoded by ligands, to predict the main-chain conformation based on ligand sequences and to chose residues for diversification which do not affect the canonical structure. It is known that, in the human  $V_{\kappa}$  domain, the L1 loop can adopt one of four canonical structures, the L2 loop has a single canonical structure and that 90% of human  $V_{\kappa}$  domains adopt one of four or five canonical structures for the L3 loop (Tomlinson *et al.* (1995) *supra*); thus, in the  $V_{\kappa}$  domain alone, different canonical structures can combine to create a range of different main-chain conformations. Given that the  $V_{\lambda}$  domain encodes a different range of canonical structures for the L1, L2 and L3 loops and that  $V_{\kappa}$  and  $V_{\lambda}$  domains can pair with any  $V_H$  domain which can encode several canonical structures for the H1 and H2 loops, the number of canonical structure combinations observed for these five loops is very large. This implies that the generation of diversity in the main-chain conformation may be essential for the production of a wide range of binding specificities. However, by constructing an antibody library based on a single known main-chain conformation it has been found, contrary to expectation, that diversity in the main-chain conformation is not required to generate sufficient diversity to target substantially all antigens. Even more surprisingly, the single main-chain conformation need not be a consensus structure - a single naturally occurring conformation can be used as the basis for an entire library. Thus, in a preferred aspect, the dual-specific ligands of the invention possess a single known main-chain conformation.

The single main-chain conformation that is chosen is preferably commonplace among molecules of the immunoglobulin superfamily type in question. A conformation is commonplace when a significant number of naturally occurring molecules are observed to adopt it. Accordingly, in a preferred aspect of the invention, the natural occurrence of the different main-chain conformations for each binding loop of an immunoglobulin domain are considered separately and then a naturally occurring variable domain is

chosen which possesses the desired combination of main-chain conformations for the different loops. If none is available, the nearest equivalent may be chosen. It is preferable that the desired combination of main-chain conformations for the different loops is created by selecting germline gene segments which encode the desired main-chain conformations. It is more preferable, that the selected germline gene segments are frequently expressed in nature, and most preferable that they are the most frequently expressed of all natural germline gene segments.

In designing dual specific ligands or libraries thereof the incidence of the different main-chain conformations for each of the six antigen binding loops may be considered separately. For H1, H2, L1, L2 and L3, a given conformation that is adopted by between 20% and 100% of the antigen binding loops of naturally occurring molecules is chosen. Typically, its observed incidence is above 35% (i.e. between 35% and 100%) and, ideally, above 50% or even above 65%. Since the vast majority of H3 loops do not have canonical structures, it is preferable to select a main-chain conformation which is commonplace among those loops which do display canonical structures. For each of the loops, the conformation which is observed most often in the natural repertoire is therefore selected. In human antibodies, the most popular canonical structures (CS) for each loop are as follows: H1 - CS 1 (79% of the expressed repertoire), H2 - CS 3 (46%), L1 - CS 2 of  $V_{\kappa}$  (39%), L2 - CS 1 (100%), L3 - CS 1 of  $V_{\kappa}$  (36%) (calculation assumes a  $\kappa:\lambda$  ratio of 70:30, Hood *et al.* (1967) *Cold Spring Harbor Symp. Quant. Biol.*, 48: 133). For H3 loops that have canonical structures, a CDR3 length (Kabat *et al.* (1991) *Sequences of proteins of immunological interest*, U.S. Department of Health and Human Services) of seven residues with a salt-bridge from residue 94 to residue 101 appears to be the most common. There are at least 16 human antibody sequences in the EMBL data library with the required H3 length and key residues to form this conformation and at least two crystallographic structures in the protein data bank which can be used as a basis for antibody modelling (2cgr and 1tet). The most frequently expressed germline gene segments that this combination of canonical structures are the  $V_H$  segment 3-23 (DP-47), the  $J_H$  segment JH4b, the  $V_{\kappa}$  segment O2/O12 (DPK9) and the  $J_{\kappa}$  segment  $J_{\kappa}1$ .  $V_H$  segments DP45 and DP38 are also suitable. These segments can therefore be used in combination as a basis to construct a library with the desired single main-chain conformation.



Alternatively, instead of choosing the single main-chain conformation based on the natural occurrence of the different main-chain conformations for each of the binding loops in isolation, the natural occurrence of combinations of main-chain conformations is used as the basis for choosing the single main-chain conformation. In the case of antibodies, for example, the natural occurrence of canonical structure combinations for any two, three, four, five or for all six of the antigen binding loops can be determined. Here, it is preferable that the chosen conformation is commonplace in naturally occurring antibodies and most preferable that it observed most frequently in the natural repertoire. Thus, in human antibodies, for example, when natural combinations of the five antigen binding loops, H1, H2, L1, L2 and L3, are considered, the most frequent combination of canonical structures is determined and then combined with the most popular conformation for the H3 loop, as a basis for choosing the single main-chain conformation.

ii. Diversification of the canonical sequence

Having selected several known main-chain conformations or, preferably a single known main-chain conformation, dual specific ligands according to the invention or libraries for use in the invention can be constructed by varying the binding site of the molecule in order to generate a repertoire with structural and/or functional diversity. This means that variants are generated such that they possess sufficient diversity in their structure and/or in their function so that they are capable of providing a range of activities.

The desired diversity is typically generated by varying the selected molecule at one or more positions. The positions to be changed can be chosen at random or are preferably selected. The variation can then be achieved either by randomisation, during which the resident amino acid is replaced by any amino acid or analogue thereof, natural or synthetic, producing a very large number of variants or by replacing the resident amino acid with one or more of a defined subset of amino acids, producing a more limited number of variants.

Various methods have been reported for introducing such diversity. Error-prone PCR (Hawkins *et al.* (1992) *J. Mol. Biol.*, 226: 889), chemical mutagenesis (Deng *et al.* (1994)

*J. Biol. Chem.*, 269: 9533) or bacterial mutator strains (Low *et al.* (1996) *J. Mol. Biol.*, 260: 359) can be used to introduce random mutations into the genes that encode the molecule. Methods for mutating selected positions are also well known in the art and include the use of mismatched oligonucleotides or degenerate oligonucleotides, with or without the use of PCR. For example, several synthetic antibody libraries have been created by targeting mutations to the antigen binding loops. The H3 region of a human tetanus toxoid-binding Fab has been randomised to create a range of new binding specificities (Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89: 4457). Random or semi-random H3 and L3 regions have been appended to germline V gene segments to produce large libraries with unmutated framework regions (Hoogenboom & Winter (1992) *J. Mol. Biol.*, 227: 381; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89: 4457; Nissim *et al.* (1994) *EMBO J.*, 13: 692; Griffiths *et al.* (1994) *EMBO J.*, 13: 3245; De Kruif *et al.* (1995) *J. Mol. Biol.*, 248: 97). Such diversification has been extended to include some or all of the other antigen binding loops (Crameri *et al.* (1996) *Nature Med.*, 2: 100; Riechmann *et al.* (1995) *Bio/Technology*, 13: 475; Morphosys, WO97/08320, supra).

Since loop randomisation has the potential to create approximately more than  $10^{15}$  structures for H3 alone and a similarly large number of variants for the other five loops, it is not feasible using current transformation technology or even by using cell free systems to produce a library representing all possible combinations. For example, in one of the largest libraries constructed to date,  $6 \times 10^{10}$  different antibodies, which is only a fraction of the potential diversity for a library of this design, were generated (Griffiths *et al.* (1994) supra).

In a preferred embodiment, only those residues which are directly involved in creating or modifying the desired function of the molecule are diversified. For many molecules, the function will be to bind a target and therefore diversity should be concentrated in the target binding site, while avoiding changing residues which are crucial to the overall packing of the molecule or to maintaining the chosen main-chain conformation.

Diversification of the canonical sequence as it applies to antibody domains

In the case of antibody dual-specific ligands, the binding site for the target is most often the antigen binding site. Thus, in a highly preferred aspect, the invention provides libraries of or for the assembly of antibody dual-specific ligands in which only those  
5 residues in the antigen binding site are varied. These residues are extremely diverse in the human antibody repertoire and are known to make contacts in high-resolution antibody/antigen complexes. For example, in L2 it is known that positions 50 and 53 are diverse in naturally occurring antibodies and are observed to make contact with the antigen. In contrast, the conventional approach would have been to diversify all the  
10 residues in the corresponding Complementarity Determining Region (CDR1) as defined by Kabat *et al.* (1991, *supra*), some seven residues compared to the two diversified in the library for use according to the invention. This represents a significant improvement in terms of the functional diversity required to create a range of antigen binding specificities.

15 In nature, antibody diversity is the result of two processes: somatic recombination of germline V, D and J gene segments to create a naive primary repertoire (so called germline and junctional diversity) and somatic hypermutation of the resulting rearranged V genes. Analysis of human antibody sequences has shown that diversity in the primary repertoire is focused at the centre of the antigen binding site whereas somatic  
20 hypermutation spreads diversity to regions at the periphery of the antigen binding site that are highly conserved in the primary repertoire (see Tomlinson *et al.* (1996) *J. Mol. Biol.*, 256: 813). This complementarity has probably evolved as an efficient strategy for searching sequence space and, although apparently unique to antibodies, it can easily be applied to other polypeptide repertoires. The residues which are varied are a subset of  
25 those that form the binding site for the target. Different (including overlapping) subsets of residues in the target binding site are diversified at different stages during selection, if desired.

In the case of an antibody repertoire, an initial 'naive' repertoire is created where some,  
30 but not all, of the residues in the antigen binding site are diversified. As used herein in this context, the term "naive" refers to antibody molecules that have no pre-determined target. These molecules resemble those which are encoded by the immunoglobulin genes of an individual who has not undergone immune diversification, as is the case with fetal

and newborn individuals, whose immune systems have not yet been challenged by a wide variety of antigenic stimuli. This repertoire is then selected against a range of antigens or epitopes. If required, further diversity can then be introduced outside the region diversified in the initial repertoire. This matured repertoire can be selected for  
5 modified function, specificity or affinity.

The invention provides two different naive repertoires of binding domains for the construction of dual specific ligands, or a naïve library of dual specific ligands, in which some or all of the residues in the antigen binding site are varied. The "primary" library  
10 mimics the natural primary repertoire, with diversity restricted to residues at the centre of the antigen binding site that are diverse in the germline V gene segments (germline diversity) or diversified during the recombination process (junctional diversity). Those residues which are diversified include, but are not limited to, H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96. In the "somatic"  
15 library, diversity is restricted to residues that are diversified during the recombination process (junctional diversity) or are highly somatically mutated). Those residues which are diversified include, but are not limited to: H31, H33, H35, H95, H96, H97, H98, L30, L31, L32, L34 and L96. All the residues listed above as suitable for diversification in these libraries are known to make contacts in one or more antibody-antigen complexes.  
20 Since in both libraries, not all of the residues in the antigen binding site are varied, additional diversity is incorporated during selection by varying the remaining residues, if it is desired to do so. It shall be apparent to one skilled in the art that any subset of any of these residues (or additional residues which comprise the antigen binding site) can be used for the initial and/or subsequent diversification of the antigen binding site.

25

In the construction of libraries for use in the invention, diversification of chosen positions is typically achieved at the nucleic acid level, by altering the coding sequence which specifies the sequence of the polypeptide such that a number of possible amino acids (all  
20 or a subset thereof) can be incorporated at that position. Using the IUPAC  
30 nomenclature, the most versatile codon is NNK, which encodes all amino acids as well as the TAG stop codon. The NNK codon is preferably used in order to introduce the required diversity. Other codons which achieve the same ends are also of use, including

the NNN codon, which leads to the production of the additional stop codons TGA and TAA.

5 A feature of side-chain diversity in the antigen binding site of human antibodies is a pronounced bias which favours certain amino acid residues. If the amino acid composition of the ten most diverse positions in each of the  $V_H$ ,  $V_K$  and  $V_L$  regions are summed, more than 76% of the side-chain diversity comes from only seven different residues, these being, serine (24%), tyrosine (14%), asparagine (11%), glycine (9%), alanine (7%), aspartate (6%) and threonine (6%). This bias towards hydrophilic residues  
10 and small residues which can provide main-chain flexibility probably reflects the evolution of surfaces which are predisposed to binding a wide range of antigens or epitopes and may help to explain the required promiscuity of antibodies in the primary repertoire.

15 Since it is preferable to mimic this distribution of amino acids, the distribution of amino acids at the positions to be varied preferably mimics that seen in the antigen binding site of antibodies. Such bias in the substitution of amino acids that permits selection of certain polypeptides (not just antibody polypeptides) against a range of target antigens is easily applied to any polypeptide repertoire. There are various methods for biasing the amino  
20 acid distribution at the position to be varied (including the use of tri-nucleotide mutagenesis, see WO97/08320), of which the preferred method, due to ease of synthesis, is the use of conventional degenerate codons. By comparing the amino acid profile encoded by all combinations of degenerate codons (with single, double, triple and quadruple degeneracy in equal ratios at each position) with the natural amino acid use it is  
25 possible to calculate the most representative codon. The codons (AGT)(AGC)T, (AGT)(AGC)C and (AGT)(AGC)(CT) - that is, DVT, DVC and DVY, respectively using IUPAC nomenclature - are those closest to the desired amino acid profile: they encode 22% serine and 11% tyrosine, asparagine, glycine, alanine, aspartate, threonine and cysteine. Preferably, therefore, libraries are constructed using either the DVT, DVC or  
30 DVY codon at each of the diversified positions.

G: Antigens capable of increasing ligand half-life

The dual specific ligands according to the invention, in one configuration thereof, are capable of binding to one or more molecules which can increase the half-life of the ligand  
5 *in vivo*. Typically, such molecules are polypeptides which occur naturally *in vivo* and which resist degradation or removal by endogenous mechanisms which remove unwanted material from the organism. For example, the molecule which increases the half-life of the organism may be selected from the following:

10 Proteins from the extracellular matrix; for example collagen, laminins, integrins and fibronectin. Collagens are the major proteins of the extracellular matrix. About 15 types of collagen molecules are currently known, found in different parts of the body, eg type I collagen (accounting for 90% of body collagen) found in bone, skin, tendon, ligaments, cornea, internal organs or type II collagen found in cartilage, intervertebral disc, notochord,  
15 vitreous humour of the eye.

Proteins found in blood, including:

Plasma proteins such as fibrin,  $\alpha$ -2 macroglobulin, serum albumin, fibrinogen A,  
20 fibrinogen B, serum amyloid protein A, heptaglobin, profilin, ubiquitin, uteroglobulin and  $\beta$ -2-microglobulin;

Enzymes and inhibitors such as plasminogen, lysozyme, cystatin C, alpha-1-antitrypsin and pancreatic trypsin inhibitor. Plasminogen is the inactive precursor of the trypsin-like  
25 serine protease plasmin. It is normally found circulating through the blood stream. When plasminogen becomes activated and is converted to plasmin, it unfolds a potent enzymatic domain that dissolves the fibrinogen fibers that entangle the blood cells in a blood clot. This is called fibrinolysis.

30 Immune system proteins, such as IgE, IgG, IgM.

Transport proteins such as retinol binding protein,  $\alpha$ -1 microglobulin.

Defensins such as beta-defensin 1, Neutrophil defensins 1,2 and 3.

Proteins found at the blood brain barrier or in neural tissues, such as melanocortin receptor, myelin, ascorbate transporter.

5

Transferrin receptor specific ligand-neuropharmaceutical agent fusion proteins (see US5977307);

10 brain capillary endothelial cell receptor, transferrin, transferrin receptor, insulin, insulin-like growth factor 1 (IGF 1) receptor, insulin-like growth factor 2 (IGF 2) receptor, insulin receptor.

Proteins localised to the kidney, such as polycystin, type IV collagen, organic anion transporter K1, Heymann's antigen.

15

Proteins localised to the liver, for example alcohol dehydrogenase, G250.

Blood coagulation factor X

$\alpha$ 1 antitrypsin

20 HNF 1 $\alpha$ 

Proteins localised to the lung, such as secretory component (binds IgA).

25 Proteins localised to the Heart, for example HSP 27. This is associated with dilated cardiomyopathy.

Proteins localised to the skin, for example keratin.

30 Bone specific proteins, such as bone morphogenic proteins (BMPs), which are a subset of the transforming growth factor  $\beta$  superfamily that demonstrate osteogenic activity. Examples include BMP-2, -4, -5, -6, -7 (also referred to as osteogenic protein (OP-1) and -8 (OP-2).

Tumour specific proteins, including human trophoblast antigen, herceptin receptor, oestrogen receptor, cathepsins eg cathepsin B (found in liver and spleen).

Disease-specific proteins, such as antigens expressed only on activated T-cells: including  
5 LAG-3 (lymphocyte activation gene), osteoprotegerin ligand (OPGL) see *Nature* 402,  
304-309; 1999, OX40 (a member of the TNF receptor family, expressed on activated T  
cells and the only costimulatory T cell molecule known to be specifically up-regulated in  
human T cell leukaemia virus type-I (HTLV-I)-producing cells.) See *J Immunol.* 2000  
*Jul 1;165(1):263-70*; Metalloproteases (associated with arthritis/cancers), including  
10 CG6512 *Drosophila*, human paraplegin, human FtsH, human AFG3L2, murine ftsH;  
angiogenic growth factors, including acidic fibroblast growth factor (FGF-1), basic  
fibroblast growth factor (FGF-2), Vascular endothelial growth factor / vascular  
permeability factor (VEGF/VPF), transforming growth factor- $\alpha$  (TGF  $\alpha$ ), tumor necrosis  
factor- $\alpha$  (TNF- $\alpha$ ), angiogenin, interleukin-3 (IL-3), interleukin-8 (IL-8), platelet-  
15 derived endothelial growth factor (PD-ECGF), placental growth factor (PlGF), midkine  
platelet-derived growth factor-BB (PDGF), fractalkine.

#### Stress proteins (heat shock proteins)

HSPs are normally found intracellularly. When they are found extracellularly, it is an  
20 indicator that a cell has died and spilled out its contents. This unprogrammed cell death  
(necrosis) only occurs when as a result of trauma, disease or injury and therefore *in vivo*,  
extracellular HSPs trigger a response from the immune system that will fight infection  
and disease. A dual specific which binds to extracellular HSP can be localised to a  
disease site.

25

#### Proteins involved in Fc transport

Brambell receptor (also known as FcRB)

This Fc receptor has two functions, both of which are potentially useful for delivery

The functions are

- 30 (1) The transport of IgG from mother to child across the placenta  
(2) the protection of IgG from degradation thereby prolonging its serum half life of  
IgG. It is thought that the receptor recycles IgG from endosome.



See Holliger *et al*, Nat Biotechnol 1997 Jul;15(7):632-6.

Ligands according to the invention may be designed to be specific for the above targets without requiring any increase in or increasing half life *in vivo*. For example, ligands  
5 according to the invention can be specific for targets selected from the foregoing which are tissue-specific, thereby enabling tissue-specific targeting of the dual specific ligand, or a dAb monomer that binds a tissue-specific therapeutically relevant target, irrespective of any increase in half-life, although this may result. Moreover, where the ligand or dAb monomer targets kidney or liver, this may redirect the ligand or dAb monomer to an  
10 alternative clearance pathway *in vivo* (for example, the ligand may be directed away from liver clearance to kidney clearance).

H: Use of multispecific ligands according to the second configuration of the invention

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Multispecific ligands according to the method of the second configuration of the present invention may be employed in *in vivo* therapeutic and prophylactic applications, *in vitro* and *in vivo* diagnostic applications, *in vitro* assay and reagent applications, and the like. For example antibody molecules may be used in antibody based assay techniques, such as  
20 ELISA techniques, according to methods known to those skilled in the art.

As alluded to above, the multispecific ligands according to the invention are of use in diagnostic, prophylactic and therapeutic procedures. Multispecific antibodies according to the invention are of use diagnostically in Western analysis and *in situ* protein detection by  
25 standard immunohistochemical procedures; for use in these applications, the ligands may be labelled in accordance with techniques known to the art. In addition, such antibody polypeptides may be used preparatively in affinity chromatography procedures, when complexed to a chromatographic support, such as a resin. All such techniques are well known to one of skill in the art.

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Diagnostic uses of the closed conformation multispecific ligands according to the invention include homogenous assays for analytes which exploit the ability of closed conformation multispecific ligands to bind two targets in competition, such that two

targets cannot bind simultaneously (a closed conformation), or alternatively their ability to bind two targets simultaneously (an open conformation).

5 A true homogenous immunoassay format has been avidly sought by manufacturers of diagnostics and research assay systems used in drug discovery and development. The main diagnostics markets include human testing in hospitals, doctor's offices and clinics, commercial reference laboratories, blood banks, and the home, non-human diagnostics (for example food testing, water testing, environmental testing, bio-defence, and veterinary testing), and finally research (including drug development; basic research and  
10 academic research).

At present all these markets utilise immunoassay systems that are built around chemiluminescent, ELISA, fluorescence or in rare cases radio-immunoassay technologies. Each of these assay formats requires a separation step (separating bound  
15 from un-bound reagents). In some cases, several separation steps are required. Adding these additional steps adds reagents and automation, takes time, and affects the ultimate outcome of the assays. In human diagnostics, the separation step may be automated, which masks the problem, but does not remove it. The robotics, additional reagents, additional incubation times, and the like add considerable cost and complexity. In drug  
20 development, such as high throughput screening, where literally millions of samples are tested at once, with very low levels of test molecule, adding additional separation steps can eliminate the ability to perform a screen. However, avoiding the separation creates too much noise in the read out. Thus, there is a need for a true homogenous format that provides sensitivities at the range obtainable from present assay formats. Advantageously,  
25 an assay possesses fully quantitative read-outs with high sensitivity and a large dynamic range. Sensitivity is an important requirement, as is reducing the amount of sample required. Both of these features are features that a homogenous system offers. This is very important in point of care testing, and in drug development where samples are precious. Heterogenous systems, as currently available in the art, require large quantities  
30 of sample and expensive reagents

Applications for homogenous assays include cancer testing, where the biggest assay is that for Prostate Specific Antigen, used in screening men for prostate cancer. Other

applications include fertility testing, which provides a series of tests for women attempting to conceive including beta-hcg for pregnancy. Tests for infectious diseases, including hepatitis, HIV, rubella, and other viruses and microorganisms and sexually transmitted diseases. Tests are used by blood banks, especially tests for HIV, hepatitis A, B, C, non A non B. Therapeutic drug monitoring tests include monitoring levels of prescribed drugs in patients for efficacy and to avoid toxicity, for example digoxin for arrhythmia, and phenobarbital levels in psychotic cases; theophylline for asthma. Diagnostic tests are moreover useful in abused drug testing, such as testing for cocaine, marijuana and the like. Metabolic tests are used for measuring thyroid function, anaemia and other physiological disorders and functions.

The homogenous immunoassay format is moreover useful in the manufacture of standard clinical chemistry assays. The inclusion of immunoassays and chemistry assays on the same instrument is highly advantageous in diagnostic testing. Suitable chemical assays include tests for glucose, cholesterol, potassium, and the like.

A further major application for homogenous immunoassays is drug discovery and development: high throughput screening includes testing combinatorial chemistry libraries versus targets in ultra high volume. Signal is detected, and positive groups then split into smaller groups, and eventually tested in cells and then animals. Homogenous assays may be used in all these types of test. In drug development, especially animal studies and clinical trials heavy use of immunoassays is made. Homogenous assays greatly accelerate and simplify these procedures. Other Applications include food and beverage testing: testing meat and other foods for E. coli, salmonella, etc; water testing, including testing at water plants for all types of contaminants including E. coli; and veterinary testing.

In a broad embodiment, the invention provides a binding assay comprising a detectable agent which is bound to a closed conformation multispecific ligand according to the invention, and whose detectable properties are altered by the binding of an analyte to said closed conformation multispecific ligand. Such an assay may be configured in several different ways, each exploiting the above properties of closed conformation multispecific ligands.

The assay relies on the direct or indirect displacement of an agent by the analyte, resulting in a change in the detectable properties of the agent. For example, where the agent is an enzyme which is capable of catalysing a reaction which has a detectable end-point, said  
5 enzyme can be bound by the ligand such as to obstruct its active site, thereby inactivating the enzyme. The analyte, which is also bound by the closed conformation multispecific ligand, displaces the enzyme, rendering it active through freeing of the active site. The enzyme is then able to react with a substrate, to give rise to a detectable event. In an alternative embodiment, the ligand may bind the enzyme outside of the active site,  
10 influencing the conformation of the enzyme and thus altering its activity. For example, the structure of the active site may be constrained by the binding of the ligand, or the binding of cofactors necessary for activity may be prevented.

The physical implementation of the assay may take any form known in the art. For  
15 example, the closed conformation multispecific ligand/enzyme complex may be provided on a test strip; the substrate may be provided in a different region of the test strip, and a solvent containing the analyte allowed to migrate through the ligand/enzyme complex, displacing the enzyme, and carrying it to the substrate region to produce a signal. Alternatively, the ligand/enzyme complex may be provided on a test stick or other solid  
20 phase, and dipped into an analyte/substrate solution, releasing enzyme into the solution in response to the presence of analyte.

Since each molecule of analyte potentially releases one enzyme molecule, the assay is quantitative, with the strength of the signal generated in a given time being dependent on  
25 the concentration of analyte in the solution.

Further configurations using the analyte in a closed conformation are possible. For example, the closed conformation multispecific ligand may be configured to bind an enzyme in an allosteric site, thereby activating the enzyme. In such an embodiment, the  
30 enzyme is active in the absence of analyte. Addition of the analyte displaces the enzyme and removes allosteric activation, thus inactivating the enzyme.

In the context of the above embodiments which employ enzyme activity as a measure of the analyte concentration, activation or inactivation of the enzyme refers to an increase or decrease in the activity of the enzyme, measured as the ability of the enzyme to catalyse a signal-generating reaction. For example, the enzyme may catalyse the conversion of an undetectable substrate to a detectable form thereof. For example, horseradish peroxidase is widely used in the art together with chromogenic or chemiluminescent substrates, which are available commercially. The level of increase or decrease of the activity of the enzyme may be between 10% and 100%, such as 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%; in the case of an increase in activity, the increase may be more than 100%, i.e. 200%, 300%, 500% or more, or may not be measurable as a percentage if the baseline activity of the inhibited enzyme is undetectable.

In a further configuration, the closed conformation multispecific ligand may bind the substrate of an enzyme/substrate pair, rather than the enzyme. The substrate is therefore unavailable to the enzyme until released from the closed conformation multispecific ligand through binding of the analyte. The implementations for this configuration are as for the configurations which bind enzyme.

Moreover, the assay may be configured to bind a fluorescent molecule, such as a fluorescein or another fluorophore, in a conformation such that the fluorescence is quenched on binding to the ligand. In this case, binding of the analyte to the ligand will displace the fluorescent molecule, thus producing a signal. Alternatives to fluorescent molecules which are useful in the present invention include luminescent agents, such as luciferin/luciferase, and chromogenic agents, including agents commonly used in immunoassays such as HRP.

Therapeutic and prophylactic uses of multispecific ligands prepared according to the invention involve the administration of ligands according to the invention to a recipient mammal, such as a human. Multi-specificity can allow antibodies to bind to multimeric antigen with great avidity. Multispecific ligands can allow the cross-linking of two antigens, for example in recruiting cytotoxic T-cells to mediate the killing of tumour cell lines.

Substantially pure ligands or binding proteins thereof, for example dAb monomers, of at least 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the mammal is a human. Once purified, partially or to homogeneity as desired, the ligands  
5 may be used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings and the like (Lefkovite and Pernis, (1979 and 1981) Immunological Methods, Volumes I and II, Academic Press, NY).

10 The ligands or binding proteins thereof, for example dAb monomers, of the present invention will typically find use in preventing, suppressing or treating inflammatory states, allergic hypersensitivity, cancer, bacterial or viral infection, and autoimmune disorders (which include, but are not limited to, Type I diabetes, asthma, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and  
15 myasthenia gravis).

In the instant application, the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the  
20 disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Animal model systems which can be used to screen the effectiveness of the antibodies or binding proteins thereof in protecting against or treating the disease are available.  
25 Methods for the testing of systemic lupus erythematosus (SLE) in susceptible mice are known in the art (Knight *et al.* (1978) *J. Exp. Med.*, 147: 1653; Reinersten *et al.* (1978) *New Eng. J. Med.*, 299: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease with soluble AchR protein from another species (Lindstrom *et al.* (1988) *Adv. Immunol.*, 42: 233). Arthritis is induced in a susceptible strain of mice by  
30 injection of Type II collagen (Stuart *et al.* (1984) *Ann. Rev. Immunol.*, 42: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has been described (Van Eden *et al.* (1988) *Nature*, 331: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron *et*

al. (1980) *J. Exp. Med.*, 152: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa *et al.* (1984) *Diabetologia*, 27: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration  
5 of myelin basic protein (see Paterson (1986) *Textbook of Immunopathology*, Mischer *et al.*, eds., Grune and Stratton, New York, pp. 179-213; McFarlin *et al.* (1973) *Science*, 179: 478; and Satoh *et al.* (1987) *J. Immunol.*, 138: 179).

Generally, the present ligands will be utilised in purified form together with  
10 pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from  
15 thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack  
20 (1982) *Remington's Pharmaceutical Sciences*, 16th Edition).

The ligands of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatinum, and immunotoxins.  
25 Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the ligands of the present invention, or even combinations of ligands according to the present invention having different specificities, such as ligands selected using different target antigens or epitopes, whether or not they are pooled prior to administration.

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The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the selected ligands thereof of the invention

can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, *via* the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

The ligands of this invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of antibody activity loss (e.g. with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted upward to compensate.

The compositions containing the present ligands or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of ligand, e.g. antibody, receptor (e.g. a T-cell receptor) or binding protein thereof *per* kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present ligands or cocktails thereof may also be administered in similar or slightly lower dosages.

Treatment performed using the compositions described herein is considered "effective" if one or more symptoms is reduced (e.g., by at least 10% or at least one point on a clinical assessment scale), relative to such symptoms present before treatment, or relative to such symptoms in an individual (human or model animal) not treated with such composition. Symptoms will obviously vary depending upon the disease or disorder targeted, but can



be measured by an ordinarily skilled clinician or technician. Such symptoms can be measured, for example, by monitoring the level of one or more biochemical indicators of the disease or disorder (e.g., levels of an enzyme or metabolite correlated with the disease, affected cell numbers, etc.), by monitoring physical manifestations (e.g.,  
5 inflammation, tumor size, etc.), or by an accepted clinical assessment scale, for example, the Expanded Disability Status Scale (for multiple sclerosis), the Irvine Inflammatory Bowel Disease Questionnaire (32 point assessment evaluates quality of life with respect to bowel function, systemic symptoms, social function and emotional status - score ranges from 32 to 224, with higher scores indicating a better quality of life), the Quality of Life  
10 Rheumatoid Arthritis Scale, or other accepted clinical assessment scale as known in the field. A sustained (e.g., one day or more, preferably longer) reduction in disease or disorder symptoms by at least 10% or by one or more points on a given clinical scale is indicative of "effective" treatment. Similarly, prophylaxis performed using a composition as described herein is "effective" if the onset or severity of one or more symptoms is  
15 delayed, reduced or abolished relative to such symptoms in a similar individual (human or animal model) not treated with the composition.

A composition containing a ligand or cocktail thereof according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration,  
20 inactivation, killing or removal of a select target cell population in a mammal. In addition, the selected repertoires of polypeptides described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the ligands, e.g. antibodies, cell-surface receptors or binding  
25 proteins thereof whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

I: Use of half-life enhanced dual-specific ligands according to the invention

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Dual-specific ligands according to the method of the present invention may be employed in *in vivo* therapeutic and prophylactic applications, *in vivo* diagnostic applications and the like.

Therapeutic and prophylactic uses of dual-specific ligands prepared according to the invention involve the administration of ligands according to the invention to a recipient mammal, such as a human. Dual specific antibodies according to the invention comprise  
5 at least one specificity for a half-life enhancing molecule; one or more further specificities may be directed against target molecules. For example, a dual-specific IgG may be specific for four epitopes, one of which is on a half-life enhancing molecule. Dual-specificity can allow antibodies to bind to multimeric antigen with great avidity. Dual-specific antibodies can allow the cross-linking of two antigens, for example in recruiting  
10 cytotoxic T-cells to mediate the killing of tumour cell lines.

Substantially pure ligands or binding proteins thereof, such as dAb monomers, of at least 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the  
15 mammal is a human. Once purified, partially or to homogeneity as desired, the ligands may be used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings and the like (Lefkovite and Pernis, (1979 and 1981) Immunological Methods, Volumes I and II, Academic Press, NY).

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The ligands of the present invention will typically find use in preventing, suppressing or treating inflammatory states, allergic hypersensitivity, cancer, bacterial or viral infection, and autoimmune disorders (which include, but are not limited to, Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease  
25 and myasthenia gravis).

In the instant application, the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the  
30 disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Animal model systems which can be used to screen the effectiveness of the dual specific ligands in protecting against or treating the disease are available. Methods for the testing of systemic lupus erythematosus (SLE) in susceptible mice are known in the art (Knight *et al.* (1978) *J. Exp. Med.*, 147: 1653; Reinersten *et al.* (1978) *New Eng. J. Med.*, 299: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease with soluble AchR protein from another species (Lindstrom *et al.* (1988) *Adv. Immunol.*, 42: 233). Arthritis is induced in a susceptible strain of mice by injection of Type II collagen (Stuart *et al.* (1984) *Ann. Rev. Immunol.*, 42: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has been described (Van Eden *et al.* (1988) *Nature*, 331: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron *et al.* (1980) *J. Exp. Med.*, 152: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa *et al.* (1984) *Diabetologia*, 27: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration of myelin basic protein (see Paterson (1986) *Textbook of Immunopathology*, Mischer *et al.*, eds., Grune and Stratton, New York, pp. 179-213; McFarlin *et al.* (1973) *Science*, 179: 478; and Satoh *et al.* (1987) *J. Immunol.*, 138: 179).

Dual specific ligands according to the invention and dAb monomers able to bind to extracellular targets involved in endocytosis (e.g. Clathrin) enable dual specific ligands to be endocytosed, enabling another specificity able to bind to an intracellular target to be delivered to an intracellular environment. This strategy requires a dual specific ligand with physical properties that enable it to remain functional inside the cell. Alternatively, if the final destination intracellular compartment is oxidising, a well folding ligand may not need to be disulphide free.

Generally, the present dual specific ligands will be utilised in purified form together with pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable

adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

5 Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) *Remington's Pharmaceutical Sciences*, 16th Edition).

10 The ligands of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cyclosporine, methotrexate, adriamycin or cisplatin, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the ligands of the present invention.

15 The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the ligands of the invention can be administered to any patient in accordance with standard techniques. The administration  
20 can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, *via* the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

25 The ligands of the invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and  
30 reconstitution can lead to varying degrees of antibody activity loss (e.g. with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted upward to compensate.

The compositions containing the present ligands or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of ligand *per* kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present ligands or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing a ligand according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal.

In addition, the selected repertoires of polypeptides described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the ligands, e.g. antibodies, cell- surface receptors or binding proteins thereof whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

The invention is further described, for the purposes of illustration only, in the following examples. As used herein, for the purposes of dAb nomenclature, human TNF $\alpha$  is referred to as TAR1 and human TNF $\alpha$  receptor 1 (p55 receptor) is referred to as TAR2.

**Example 1. Selection of a dual specific scFv antibody (K8) directed against human serum albumin (HSA) and  $\beta$ -galactosidase ( $\beta$ -gal)**

This example explains a method for making a dual specific antibody directed against  $\beta$ -gal and HSA in which a repertoire of V $\kappa$  variable domains linked to a germline (dummy)

V<sub>H</sub> domain is selected for binding to β-gal and a repertoire of V<sub>H</sub> variable domains linked to a germline (dummy) V<sub>κ</sub> domain is selected for binding to HSA. The selected variable V<sub>H</sub> HSA and V<sub>κ</sub> β-gal domains are then combined and the antibodies selected for binding to β-gal and HSA. HSA is a half-life increasing protein found in human blood.

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Four human phage antibody libraries were used in this experiment.

Library 1	Germline V <sub>κ</sub> /DVT V <sub>H</sub>	8.46 x 10 <sup>7</sup>
Library 2	Germline V <sub>κ</sub> /NNK V <sub>H</sub>	9.64 x 10 <sup>7</sup>
10 Library 3	Germline V <sub>H</sub> /DVT V <sub>κ</sub>	1.47 x 10 <sup>8</sup>
Library 4	Germline V <sub>H</sub> /NNK V <sub>κ</sub>	1.45 x 10 <sup>8</sup>

All libraries are based on a single human framework for V<sub>H</sub> (V3-23/DP47 and J<sub>H</sub>4b) and V<sub>κ</sub> (O12/O2/DPK9 and J<sub>κ</sub>1) with side chain diversity incorporated in complementarity determining regions (CDR2 and CDR3).

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Library 1 and Library 2 contain a dummy V<sub>κ</sub> sequence, whereas the sequence of V<sub>H</sub> is diversified at positions H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97 and H98 (DVT or NNK encoded, respectively) (Figure 1). Library 3 and Library 4 contain a dummy V<sub>H</sub> sequence, whereas the sequence of V<sub>κ</sub> is diversified at positions L50, L53, L91, L92, L93, L94 and L96 (DVT or NNK encoded, respectively) (Figure 1). The libraries are in phagemid pIT2/ScFv format (Figure 2) and have been preselected for binding to generic ligands, Protein A and Protein L, so that the majority of clones in the unselected libraries are functional. The sizes of the libraries shown above correspond to the sizes after preselection. Library 1 and Library 2 were mixed prior to selections on antigen to yield a single V<sub>H</sub>/dummy V<sub>κ</sub> library and Library 3 and Library 4 were mixed to form a single V<sub>κ</sub>/dummy V<sub>H</sub> library.

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Three rounds of selections were performed on β-gal using V<sub>κ</sub>/dummy V<sub>H</sub> library and three rounds of selections were performed on HSA using V<sub>H</sub>/dummy V<sub>κ</sub> library. In the

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case of  $\beta$ -gal the phage titres went up from  $1.1 \times 10^6$  in the first round to  $2.0 \times 10^8$  in the third round. In the case of HSA the phage titres went up from  $2 \times 10^4$  in the first round to  $1.4 \times 10^9$  in the third round. The selections were performed as described by Griffith *et al.*, (1993), except that KM13 helper phage (which contains a pIII protein with a protease cleavage site between the D2 and D3 domains) was used and phage were eluted with 1 mg/ml trypsin in PBS. The addition of trypsin cleaves the pIII proteins derived from the helper phage (but not those from the phagemid) and elutes bound scFv-phage fusions by cleavage in the c-myc tag (Figure 2), thereby providing a further enrichment for phages expressing functional scFvs and a corresponding reduction in background (Kristensen & Winter, *Folding & Design* 3: 321-328, Jul 9, 1998). Selections were performed using immunotubes coated with either HSA or  $\beta$ -gal at 100 $\mu$ g/ml concentration.

To check for binding, 24 colonies from the third round of each selection were screened by monoclonal phage ELISA. Phage particles were produced as described by Harrison *et al.*, *Methods Enzymol.* 1996;267:83-109. 96-well ELISA plates were coated with 100 $\mu$ l of HSA or  $\beta$ -gal at 10 $\mu$ g/ml concentration in PBS overnight at 4°C. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. A selection of clones gave ELISA signals of greater than 1.0 with 50 $\mu$ l supernatant.

Next, DNA preps were made from  $V_H$ /dummy  $V_K$  library selected on HSA and from  $V_K$ /dummy  $V_H$  library selected on  $\beta$ -gal using the QIAprep Spin Miniprep kit (Qiagen). To access most of the diversity, DNA preps were made from each of the three rounds of selections and then pulled together for each of the antigens. DNA preps were then digested with SalI/NotI overnight at 37°C. Following gel purification of the fragments,  $V_K$  chains from the  $V_K$ /dummy  $V_H$  library selected on  $\beta$ -gal were ligated in place of a dummy  $V_K$  chain of the  $V_H$ /dummy  $V_K$  library selected on HSA creating a library of  $3.3 \times 10^9$  clones.

This library was then either selected on HSA (first round) and  $\beta$ -gal (second round), HSA/ $\beta$ -gal selection, or on  $\beta$ -gal (first round) and HSA (second round),  $\beta$ -gal/HSA

selection. Selections were performed as described above. In each case after the second round 48 clones were tested for binding to HSA and  $\beta$ -gal by the monoclonal phage ELISA (as described above) and by ELISA of the soluble scFv fragments. Soluble antibody fragments were produced as described by Harrison *et al.*, (1996), and standard  
5 ELISA protocol was followed Hoogenboom *et al.* (1991) Nucleic Acids Res., 19: 4133, except that 2% Tween/PBS was used as a blocking buffer and bound scFvs were detected with Protein L-HRP. Three clones (E4, E5 and E8) from the HSA/ $\beta$ -gal selection and two clones (K8 and K10) from the  $\beta$ -gal/HSA selection were able to bind both antigens. scFvs from these clones were PCR amplified and sequenced as described by Ignatovich *et al.*,  
10 (1999) J Mol Biol 1999 Nov 26;294(2):457-65, using the primers LMB3 and pHENseq. Sequence analysis revealed that all clones were identical. Therefore, only one clone encoding a dual specific antibody (K8) was chosen for further work (Figure 3).

15 **Example 2. Characterisation of the binding properties of the K8 antibody.**

Firstly, the binding properties of the K8 antibody were characterised by the monoclonal phage ELISA. A 96-well plate was coated with 100 $\mu$ l of HSA and  $\beta$ -gal alongside with alkaline phosphatase (APS), bovine serum albumin (BSA), peanut agglutinin, lysozyme  
20 and cytochrome c (to check for cross-reactivity) at 10 $\mu$ g/ml concentration in PBS overnight at 4°C. The phagemid from K8 clone was rescued with KM13 as described by Harrison *et al.*, (1996) and the supernatant (50 $\mu$ l) containing phage assayed directly. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. The dual specific K8 antibody was found to  
25 bind to HSA and  $\beta$ -gal when displayed on the surface of the phage with absorbance signals greater than 1.0 (Figure 4). Strong binding to BSA was also observed (Figure 4). Since HSA and BSA are 76% homologous on the amino acid level, it is not surprising that K8 antibody recognised both of these structurally related proteins. No cross-reactivity with other proteins was detected (Figure 4).

30

Secondly, the binding properties of the K8 antibody were tested in a soluble scFv ELISA. Production of the soluble scFv fragment was induced by IPTG as described by Harrison *et al.*, (1996). To determine the expression levels of K8 scFv, the soluble antibody



fragments were purified from the supernatant of 50ml inductions using Protein A-Sepharose columns as described by Harlow and Lane, *Antibodies: a Laboratory Manual*, (1988) Cold Spring Harbor. OD<sub>280</sub> was then measured and the protein concentration calculated as described by Sambrook *et al.*, (1989). K8 scFv was produced in supernatant  
5 at 19mg/l.

A soluble scFv ELISA was then performed using known concentrations of the K8 antibody fragment. A 96-well plate was coated with 100µl of HSA, BSA and β-gal at 10µg/ml and 100µl of Protein A at 1µg/ml concentration. 50µl of the serial dilutions of  
10 the K8 scFv was applied and the bound antibody fragments were detected with Protein L-HRP. ELISA results confirmed the dual specific nature of the K8 antibody (Figure 5).

To confirm that binding to β-gal is determined by the V<sub>K</sub> domain and binding to HSA/BSA by the V<sub>H</sub> domain of the K8 scFv antibody, the V<sub>K</sub> domain was cut out from  
15 K8 scFv DNA by SalI/NotI digestion and ligated into a SalI/NotI digested pIT2 vector containing dummy V<sub>H</sub> chain (Figures 1 and 2). Binding characteristics of the resulting clone K8V<sub>K</sub>/dummy V<sub>H</sub> were analysed by soluble scFv ELISA. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al.*, (1996) and the supernatant (50µl) containing scFvs assayed directly. Soluble scFv ELISA was performed  
20 as described in Example 1 and the bound scFvs were detected with Protein L-HRP. The ELISA results revealed that this clone was still able to bind β-gal, whereas binding to BSA was abolished (Figure 6).

**Example 3. Selection of single V<sub>H</sub> domain antibodies against antigens A and B and single  
25 V<sub>K</sub> domain antibodies directed against antigens C and D.**

This example describes a method for making single V<sub>H</sub> domain antibodies directed against antigens A and B and single V<sub>K</sub> domain antibodies directed against antigens C and D by selecting repertoires of virgin single antibody variable domains for binding to  
30 these antigens in the absence of the complementary variable domains.

Selections and characterisation of the binding clones is performed as described previously (see Example 5, PCT/GB 02/003014). Four clones are chosen for further work:

- 5    VH1 – Anti A  $V_H$
- VH2 – Anti B  $V_H$
- VK1 – Anti C  $V_K$
- VK2 – Anti D  $V_K$

- 10   The procedures described above in Examples 1-3 may be used, in a similar manner as that described, to produce dimer molecules comprising combinations of  $V_H$  domains (i.e.,  $V_H$ - $V_H$  ligands) and combinations of  $V_L$  domains ( $V_L$ - $V_L$  ligands).

- Example 4. Creation and characterisation of the dual specific ScFv antibodies**
- 15   **(VH1/VH2 directed against antigens A and B and VK1/VK2 directed against antigens C and D).**

- 20   This example demonstrates that dual specific ScFv antibodies (VH1/VH2 directed against antigens A and B and VK1/VK2 directed against antigens C and D) could be created by combining  $V_K$  and  $V_H$  single domains selected against respective antigens in a ScFv vector.

- 25   To create dual specific antibody VH1/VH2, VH1 single domain is excised from variable domain vector 1 (Figure 7) by *NcoI/XhoI* digestion and ligated into *NcoI/XhoI* digested variable domain vector 2 (Figure 7) to create VH1/ variable domain vector 2. VH2 single domain is PCR amplified from variable domain vector 1 using primers to introduce *SaII* restriction site to the 5' end and *NotI* restriction site to the 3' end. The PCR product is then digested with *SaII/NotI* and ligated into *SaII/NotI* digested VH1/ variable domain vector 2 to create VH1/VH2/ variable domain vector 2.

30

VK1/VK2/ variable domain vector 2 is created in a similar way. The dual specific nature of the produced VH1/VH2 ScFv and VK1/VK2 ScFv is tested in a soluble ScFv ELISA

as described previously (see Example 6, PCT/GB 02/003014). Competition ELISA is performed as described previously (see Example 8, PCT/GB 02/003014).

Possible outcomes:

- 5 -VH1/VH2 ScFv is able to bind antigens A and B simultaneously
- VK1/VK2 ScFv is able to bind antigens C and D simultaneously
- VH1/VH2 ScFv binding is competitive (when bound to antigen A, VH1/VH2 ScFv cannot bind to antigen B)
- VK1/VK2 ScFv binding is competitive (when bound to antigen C, VK1/VK2 ScFv cannot bind to antigen D)

**Example 5. Construction of dual specific VH1/VH2 Fab and VK1/VK2 Fab and analysis of their binding properties.**

- 15 To create VH1/VH2 Fab, VH1 single domain is ligated into *NcoI/XhoI* digested CH vector (Figure 8) to create VH1/CH and VH2 single domain is ligated into *SalI/NorI* digested CK vector (Figure 9) to create VH2/CK. Plasmid DNA from VH1/CH and VH2/CK is used to co-transform competent *E. coli* cells as described previously (see Example 8, PCT/GB02/003014).

20

The clone containing VH1/CH and VH2/CK plasmids is then induced by IPTG to produce soluble VH1/VH2 Fab as described previously (see Example 8, PCT/GB 02/003014).

- 25 VK1/VK2 Fab is produced in a similar way.

Binding properties of the produced Fabs are tested by competition ELISA as described previously (see Example 8, PCT/GB 02/003014).

- 30 Possible outcomes:

- VH1/VH2 Fab is able to bind antigens A and B simultaneously
- VK1/VK2 Fab is able to bind antigens C and D simultaneously

-VH1/VH2 Fab binding is competitive (when bound to antigen A, VH1/VH2 Fab cannot bind to antigen B)

-VK1/VK2 Fab binding is competitive (when bound to antigen C, VK1/VK2 Fab cannot bind to antigen D)

5

## Example 6

### Chelating dAb Dimers

#### 10 Summary

VH and VK homo-dimers are created in a dAb-linker-dAb format using flexible polypeptide linkers. Vectors were created in the dAb linker-dAb format containing glycine-serine linkers of different lengths 3U:(Gly<sub>4</sub>Ser)<sub>3</sub>, 5U:(Gly<sub>4</sub>Ser)<sub>5</sub>, 7U:(Gly<sub>4</sub>Ser)<sub>7</sub>. Dimer libraries were created using guiding dAbs upstream of the linker: TAR1-5 (VK),  
15 TAR1-27(VK), TAR2-5(VH) or TAR2-6(VK) and a library of corresponding second dAbs after the linker. Using this method, novel dimeric dAbs were selected. The effect of dimerisation on antigen binding was determined by ELISA and BIAcore studies and in cell neutralisation and receptor binding assays. Dimerisation of both TAR1-5 and TAR1-27 resulted in significant improvement in binding affinity and neutralisation levels.

20

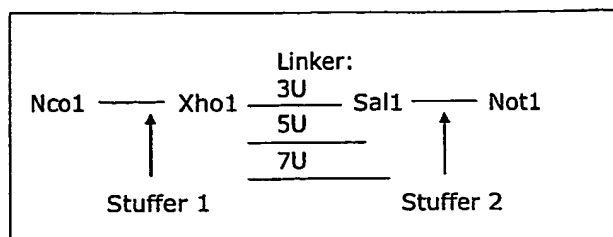
#### 1.0 Methods

##### 1.1 Library generation

##### 1.1.1 Vectors

pEDA3U, pEDA5U and pEDA7U vectors were designed to introduce different linker  
25 lengths compatible with the dAb-linker-dAb format. For pEDA3U, sense and anti-sense 73-base pair oligo linkers were annealed using a slow annealing program (95°C-5mins, 80°C-10mins, 70°C-15mins, 56°C-15mins, 42°C until use) in buffer containing 0.1MNaCl, 10mM Tris-HCl pH7.4 and cloned using the *Xho*I and *Not*I restriction sites. The linkers encompassed 3 (Gly<sub>4</sub>Ser) units and a stuffer region housed between *Sal*I and  
30 *Not*I cloning sites (scheme 1). In order to reduce the possibility of monomeric dAbs being selected for by phage display, the stuffer region was designed to include 3 stop codons, a *Sac*I restriction site and a frame shift mutation to put the region out of frame when no second dAb was present. For pEDA5U and 7U due to the length of the linkers

required, overlapping oligo-linkers were designed for each vector, annealed and elongated using Klenow. The fragment was then purified and digested using the appropriate enzymes before cloning using the *XhoI* and *NotI* restriction sites.



Scheme 1

### 1.1.2 Library preparation

The N-terminal V gene corresponding to the guiding dAb was cloned upstream of the linker using *NcoI* and *XhoI* restriction sites. VH genes have existing compatible sites, however cloning VK genes required the introduction of suitable restriction sites. This was achieved by using modifying PCR primers (VK-DLIBF: 5' cggccatggcgtaacggacat; VKXho1R: 5' atgtgcgctcgagcggttgattt 3') in 30 cycles of PCR amplification using a 2:1 mixture of SuperTaq (HTBiotechnology Ltd) and *pfu* turbo (Stratagene). This maintained the *NcoI* site at the 5' end while destroying the adjacent *SalI* site and introduced the *XhoI* site at the 3' end. 5 guiding dAbs were cloned into each of the 3 dimer vectors: TAR1-5 (VK), TAR1-27(VK), TAR2-5(VH), TAR2-6(VK) and TAR2-7(VK). All constructs were verified by sequence analysis.

Having cloned the guiding dAbs upstream of the linker in each of the vectors (pEDA3U, 5U and 7U): TAR1-5 (VK), TAR1-27(VK), TAR2-5(VH) or TAR2-6(VK) a library of corresponding second dAbs were cloned after the linker. To achieve this, the complimentary dAb libraries were PCR amplified from phage recovered from round 1 selections of either a VK library against Human TNF $\alpha$  (at approximately  $1 \times 10^6$  diversity after round 1) when TAR1-5 or TAR1-27 are the guiding dAbs, or a VH or VK library against human p55 TNF receptor (both at approximately  $1 \times 10^5$  diversity after round 1) when TAR2-5 or TAR2-6 respectively are the guiding dAbs. For VK libraries PCR amplification was conducted using primers in 30 cycles of PCR amplification using a 2:1 mixture of SuperTaq and *pfu* turbo. VH libraries were PCR amplified using primers in

order to introduce a *Sall* restriction site at the 5' end of the gene. The dAb library PCRs were digested with the appropriate restriction enzymes, ligated into the corresponding vectors down stream of the linker, using *Sall/NotI* restriction sites and electroporated into freshly prepared competent TG1 cells.

5

The titres achieved for each library are as follows:

TAR1-5: pEDA3U =  $4 \times 10^8$ , pEDA5U =  $8 \times 10^7$ , pEDA7U =  $1 \times 10^8$

TAR1-27: pEDA3U =  $6.2 \times 10^8$ , pEDA5U =  $1 \times 10^8$ , pEDA7U =  $1 \times 10^9$

TAR2h-5: pEDA3U =  $4 \times 10^7$ , pEDA5U =  $2 \times 10^8$ , pEDA7U =  $8 \times 10^7$

10 TAR2h-6: pEDA3U =  $7.4 \times 10^8$ , pEDA5U =  $1.2 \times 10^8$ , pEDA7U =  $2.2 \times 10^8$

## 1.2 Selections

### 1.2.1 TNF $\alpha$

Selections were conducted using human TNF $\alpha$  passively coated on immunotubes.

15 Briefly, Immunotubes are coated overnight with 1-4mls of the required antigen. The immunotubes were then washed 3 times with PBS and blocked with 2% milk powder in PBS for 1-2hrs and washed a further 3 times with PBS. The phage solution is diluted in 2% milk powder in PBS and incubated at room temperature for 2hrs. The tubes are then washed with PBS and the phage eluted with 1mg/ml trypsin-PBS. Three selection  
20 strategies were investigated for the TAR1-5 dimer libraries. The first round selections were carried out in immunotubes using human TNF $\alpha$  coated at 1 $\mu$ g/ml or 20 $\mu$ g/ml with 20 washes in PBS 0.1% Tween. TG1 cells are infected with the eluted phage and the titres are determined (eg, Marks et al J Mol Biol. 1991 Dec 5;222(3):581-97, Richmann et al Biochemistry. 1993 Aug 31;32(34):8848-55).

25

The titres recovered were:

pEDA3U =  $2.8 \times 10^7$  (1 $\mu$ g/ml TNF)  $1.5 \times 10^8$  (20 $\mu$ g/ml TNF),

pEDA5U =  $1.8 \times 10^7$  (1 $\mu$ g/ml TNF),  $1.6 \times 10^8$  (20 $\mu$ g/ml TNF)

pEDA7U =  $8 \times 10^6$  (1 $\mu$ g/ml TNF),  $7 \times 10^7$  (20 $\mu$ g/ml TNF).

30

The second round selections were carried out using 3 different methods.

1. In immunotubes, 20 washes with overnight incubation followed by a further 10 washes.

2. In immunotubes, 20 washes followed by 1hr incubation at RT in wash buffer with (1µg/ml TNFα) and 10 further washes.
3. Selection on streptavidin beads using 33 pmoles biotinylated human TNFα (Henderikx *et al.*, 2002, *Selection of antibodies against biotinylated antigens*. Antibody Phage Display : Methods and protocols, Ed. O'Brien and Atkin, Humana Press). Single clones from round 2 selections were picked into 96 well plates and crude supernatant preps were made in 2ml 96 well plate format.

	Round 1 Human TNFαimmuno tube coating concentration	Round 2 selection method 1	Round 2 selection method 2	Round 2 selection method 3
pEDA3U	1µg/ml	$1 \times 10^9$	$1.8 \times 10^9$	$2.4 \times 10^{10}$
pEDA3U	20µg/ml	$6 \times 10^9$	$1.8 \times 10^{10}$	$8.5 \times 10^{10}$
pEDA5U	1µg/ml	$9 \times 10^8$	$1.4 \times 10^9$	$2.8 \times 10^{10}$
pEDA5U	20µg/ml	$9.5 \times 10^9$	$8.5 \times 10^9$	$2.8 \times 10^{10}$
pEDA7U	1µg/ml	$7.8 \times 10^8$	$1.6 \times 10^8$	$4 \times 10^{10}$
pEDA7U	20µg/ml	$1 \times 10^{10}$	$8 \times 10^9$	$1.5 \times 10^{10}$

- 10 For TAR1-27, selections were carried out as described previously with the following modifications. The first round selections were carried out in immunotubes using human TNFα coated at 1µg/ml or 20µg/ml with 20 washes in PBS 0.1%Tween. The second round selections were carried out in immunotubes using 20 washes with overnight incubation followed by a further 20 washes. Single clones from round 2 selections were
- 15 picked into 96 well plates and crude supernatant preps were made in 2ml 96 well plate format.

TAR1-27 titres are as follows:

	Human TNF $\alpha$ immunotube coating conc	Round 1	Round 2
pEDA3U	1 $\mu$ g/ml	$4 \times 10^9$	$6 \times 10^9$
pEDA3U	20 $\mu$ g/ml	$5 \times 10^9$	$4.4 \times 10^{10}$
pEDA5U	1 $\mu$ g/ml	$1.5 \times 10^9$	$1.9 \times 10^{10}$
pEDA5U	20 $\mu$ g/ml	$3.4 \times 10^9$	$3.5 \times 10^{10}$
pEDA7U	1 $\mu$ g/ml	$2.6 \times 10^9$	$5 \times 10^9$
pEDA7U	20 $\mu$ g/ml	$7 \times 10^9$	$1.4 \times 10^{10}$

### 1.2.2 TNF RECEPTOR 1 (p55 RECEPTOR; TAR2)

Selections were conducted as described previously for the TAR2h-5 libraries only. 3 rounds of selections were carried out in immunotubes using either 1 $\mu$ g/ml human p55

- 5 TNF receptor or 10 $\mu$ g/ml human p55 TNF receptor with 20 washes in PBS 0.1%Tween with overnight incubation followed by a further 20 washes. Single clones from round 2 and 3 selections were picked into 96 well plates and crude supernatant preps were made in 2ml 96 well plate format.

- 10 TAR2h-5 titres are as follows:

	Round 1 human p55 TNF receptor immunotube coating concentration	Round 1	Round 2	Round 3
pEDA3U	1 $\mu$ g/ml	$2.4 \times 10^6$	$1.2 \times 10^7$	$1.9 \times 10^9$
pEDA3U	10 $\mu$ g/ml	$3.1 \times 10^7$	$7 \times 10^7$	$1 \times 10^9$
pEDA5U	1 $\mu$ g/ml	$2.5 \times 10^6$	$1.1 \times 10^7$	$5.7 \times 10^8$
pEDA5U	10 $\mu$ g/ml	$3.7 \times 10^7$	$2.3 \times 10^8$	$2.9 \times 10^9$
pEDA7U	1 $\mu$ g/ml	$1.3 \times 10^6$	$1.3 \times 10^7$	$1.4 \times 10^9$
pEDA7U	10 $\mu$ g/ml	$1.6 \times 10^7$	$1.9 \times 10^7$	$3 \times 10^{10}$

### 1.3 Screening

- Single clones from round 2 or 3 selections were picked from each of the 3U, 5U and 7U  
 15 libraries from the different selections methods, where appropriate. Clones were grown in 2xTY with 100 $\mu$ g/ml ampicillin and 1% glucose overnight at 37°C. A 1/100 dilution of this culture was inoculated into 2mls of 2xTY with 100 $\mu$ g/ml ampicillin and 0.1% glucose in 2ml, 96 well plate format and grown at 37°C shaking until OD600 was



approximately 0.9. The culture was then induced with 1mM IPTG overnight at 30°C. The supernatants were clarified by centrifugation at 4000rpm for 15 mins in a sorval plate centrifuge. The supernatant preps the used for initial screening.

### 5 1.3.1 ELISA

Binding activity of dimeric recombinant proteins was compared to monomer by Protein A/L ELISA or by antigen ELISA. Briefly, a 96 well plate is coated with antigen or Protein A/L overnight at 4°C. The plate washed with 0.05% Tween-PBS, blocked for 2hrs with 2% Tween-PBS. The sample is added to the plate incubated for 1 hr at room  
10 temperature. The plate is washed and incubated with the secondary reagent for 1hr at room temperature. The plate is washed and developed with TMB substrate. Protein A/L-HRP or India-HRP was used as a secondary reagent. For antigen ELISAs, the antigen concentrations used were 1µg/ml in PBS for Human TNFα and human THF receptor 1. Due to the presence of the guiding dAb in most cases dimers gave a positive ELISA  
15 signal therefore off rate determination was examined by BIAcore.

### 1.3.2 BIAcore

BIAcore analysys was conducted for TAR1-5 and TAR2h-5 clones. For screening, Human TNFα was coupled to a CM5 chip at high density (approximately 10000 RUs).  
20 50 µl of Human TNFα(50 µg/ml) was coupled to the chip at 5µl/min in acetate buffer - pH5.5. Regeneration of the chip following analysis using the standard methods is not possible due to the instability of Human TNFα, therefore after each sample was analysed, the chip was washed for 10mins with buffer.

For TAR1-5, clones supernatants from the round 2 selection were screened by BIAcore.

25 48 clones were screened from each of the 3U, 5U and 7U libraries obtained using the following selection methods:

R1: 1µg/ml human TNFα immunotube, R2 1µg/ml human TNFα immunotube, overnight wash.

R1: 20µg/ml human TNFα immunotube, R2 20µg/ml human TNFα immunotube,  
30 overnight wash.

R1: 1µg/ml human TNFα immunotube, R2 33 pmoles biotinylated human TNFα on beads.

R1: 20µg/ml human TNFα immunotube, R2 33 pmoles biotinylated human TNFα beads.

For screening, human p55 TNF receptor was coupled to a CM5 chip at high density (approximately 4000 RUs). 100 µl of human p55 TNF receptor (10 µg/ml) was coupled to the chip at 5µl/min in acetate buffer - pH5.5. Standard regeneration conditions were examined ( glycine pH2 or pH3) but in each case antigen was removed from the surface of the chip therefore as with TNFα, therefore after each sample was analysed, the chip was washed for 10mins with buffer.

For TAR2-5, clones supernatants from the round 2 selection were screened.

48 clones were screened from each of the 3U, 5U and 7U libraries, using the following selection methods:

R1: 1µg/ml human p55 TNF receptor immunotube, R2 1µg/ml human p55 TNF receptor immunotube, overnight wash.

R1: 10µg/ml human p55 TNF receptor immunotube, R2 10µg/ml human p55 TNF receptor immunotube, overnight wash.

### 1.3.3 Receptor and Cell Assays

The ability of the dimers to neutralise in the receptor assay was conducted as follows:

#### *Receptor binding*

Anti-TNF dAbs were tested for the ability to inhibit the binding of TNF to recombinant TNF receptor 1 (p55). Briefly, Maxisorp plates were incubated overnight with 30mg/ml anti-human Fc mouse monoclonal antibody (Zymed, San Francisco, USA). The wells were washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 and then blocked with 1% BSA in PBS before being incubated with 100ng/ml TNF receptor 1 Fc fusion protein (R&D Systems, Minneapolis, USA). Anti-TNF dAb was mixed with TNF which was added to the washed wells at a final concentration of 10ng/ml. TNF binding was detected with 0.2mg/ml biotinylated anti-TNF antibody (HyCult biotechnology, Uben, Netherlands) followed by 1 in 500 dilution of horse radish peroxidase labelled streptavidin (Amersham Biosciences, UK) and then incubation with TMB substrate (KPL, Gaithersburg, USA). The reaction was stopped by the addition of HCl and the absorbance was read at 450nm. Anti-TNF dAb activity lead to a decrease in TNF binding and therefore a decrease in absorbance compared with the TNF only control.

*L929 Cytotoxicity Assay*

Anti-TNF dAbs were also tested for the ability to neutralise the cytotoxic activity of TNF on mouse L929 fibroblasts (Evans, T. (2000) *Molecular Biotechnology* 15, 243-248). Briefly, L929 cells plated in microtitre plates were incubated overnight with anti-TNF dAb, 100pg/ml TNF and 1mg/ml actinomycin D (Sigma, Poole, UK). Cell viability was measured by reading absorbance at 490nm following an incubation with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, USA). Anti-TNF dAb activity lead to a decrease in TNF cytotoxicity and therefore an increase in absorbance compared with the TNF only control.

In the initial screen, supernatants prepared for BIAcore analysis, described above, were also used in the receptor assay. Further analysis of selected dimers was also conducted in the receptor and cell assays using purified proteins.

*HeLa IL-8 assay*

Anti-TNFR1 or anti-TNF alpha dAbs were tested for the ability to neutralise the induction of IL-8 secretion by TNF in HeLa cells (method adapted from that of Akeson, L. et al (1996) *Journal of Biological Chemistry* 271, 30517-30523, describing the induction of IL-8 by IL-1 in HUVEC; here we look at induction by human TNF alpha and we use HeLa cells instead of the HUVEC cell line). Briefly, HeLa cells plated in microtitre plates were incubated overnight with dAb and 300pg/ml TNF. Post incubation the supernatant was aspirated off the cells and IL-8 concentration measured via a sandwich ELISA (R&D Systems). Anti-TNFR1 dAb activity lead to a decrease in IL-8 secretion into the supernatant compared with the TNF only control.

The L929 assay is used throughout the following experiments; however, the use of the HeLa IL-8 assay is preferred to measure anti-TNF receptor 1 (p55) ligands; the presence of mouse p55 in the L929 assay poses certain limitations in its use.

**1.4 Sequence analysis**

Dimers that proved to have interesting properties in the BIAcore and the receptor assay screens were sequenced. Sequences are detailed in the sequence listing.

## 1.5 Formatting

### 1.5.1 TAR1-5-19 dimers

The TAR1-5 dimers that were shown to have good neutralisation properties were re-formatted and analysed in the cell and receptor assays. The TAR1-5 guiding dab was substituted with the affinity matured clone TAR1-5-19. To achieve this TAR1-5 was cloned out of the individual dimer pair and substituted with TAR1-5-19 that had been amplified by PCR. In addition, TAR1-5-19 homodimers were also constructed in the 3U, 5U and 7U vectors. The N terminal copy of the gene was amplified by PCR and cloned as described above and the C-terminal gene fragment was cloned using existing *Sall* and *NotI* restriction sites.

### 1.5.2 Mutagenesis

The amber stop codon present in dAb2, one of the C-terminal dAbs in the TAR1-5 dimer pairs was mutated to a glutamine by site-directed mutagenesis.

### 1.5.3 Fabs

The dimers containing TAR1-5 or TAR1-5-19 were re-formatted into Fab expression vectors. dAbs were cloned into expression vectors containing either the CK or CH genes using *SfiI* and *NotI* restriction sites and verified by sequence analysis. The CK vector is derived from a pUC based ampicillin resistant vector and the CH vector is derived from a pACYC chloramphenicol resistant vector. For Fab expression the dAb-CH and dAb-CK constructs were co-transformed into HB2151 cells and grown in 2xTY containing 0.1% glucose, 100µg/ml ampicillin and 10µg/ml chloramphenicol.

### 1.5.3 Hinge dimerisation

Dimerisation of dAbs via cystine bond formation was examined. A short sequence of amino acids EPKSGDKTHTCPPCP a modified form of the human IgGC1 hinge was engineered at the C terminal region on the dAb. An oligo linker encoding for this sequence was synthesised and annealed, as described previously. The linker was cloned into the pEDA vector containing TAR1-5-19 using *XhoI* and *NotI* restriction sites. Dimerisation occurs *in situ* in the periplasm.

## 1.6 Expression and purification

### 1.6.1 Expression

Supernatants were prepared in the 2ml, 96-well plate format for the initial screening as described previously. Following the initial screening process selected dimers were analysed further. Dimer constructs were expressed in TOP10F' or HB2151 cells as  
5 supernatants. Briefly, an individual colony from a freshly streaked plate was grown overnight at 37°C in 2xTY with 100µg/ml ampicillin and 1% glucose. A 1/100 dilution of this culture was inoculated into 2xTY with 100µg/ml ampicillin and 0.1% glucose and grown at 37°C shaking until OD600 was approximately 0.9. The culture was then induced with 1mM IPTG overnight at 30°C. The cells were removed by centrifugation and the  
10 supernatant purified with protein A or L agarose.

Fab and cysteine hinge dimers were expressed as periplasmic proteins in HB2152 cells. A 1/100 dilution of an overnight culture was inoculated into 2xTY with 0.1% glucose and the appropriate antibiotics and grown at 30°C shaking until OD600 was approximately 0.9. The culture was then induced with 1mM IPTG for 3-4 hours at 25°C.  
15 The cells were harvested by centrifugation and the pellet resuspended in periplasmic preparation buffer (30mM Tris-HCl pH8.0, 1mM EDTA, 20% sucrose). Following centrifugation the supernatant was retained and the pellet resuspended in 5mM MgSO<sub>4</sub>. The supernatant was harvested again by centrifugation, pooled and purified.

### 20 1.6.2 Protein A/L purification

Optimisation of the purification of dimer proteins from Protein L agarose (Affitech, Norway) or Protein A agarose (Sigma, UK) was examined. Protein was eluted by batch or by column elution using a peristaltic pump. Three buffers were examined 0.1M Phosphate-citrate buffer pH2.6, 0.2M Glycine pH2.5 and 0.1M Glycine pH2.5. The  
25 optimal condition was determined to be under peristaltic pump conditions using 0.1M Glycine pH2.5 over 10 column volumes. Purification from protein A was conducted peristaltic pump conditions using 0.1M Glycine pH2.5.

### 1.6.3 FPLC purification

30 Further purification was carried out by FPLC analysis on the AKTA Explorer 100 system (Amersham Biosciences Ltd). TAR1-5 and TAR1-5-19 dimers were fractionated by cation exchange chromatography (1ml Resource S – Amersham Biosciences Ltd) eluted with a 0-1M NaCl gradient in 50mM acetate buffer pH4. Hinge dimers were purified by

ion exchange (1ml Resource Q Amersham Biosciences Ltd) eluted with a 0-1M NaCl gradient in 25mMTris HCl pH 8.0. Fabs were purified by size exclusion chromatography using a superose 12 (Amersham Biosciences Ltd ) column run at a flow rate of 0.5ml/min in PBS with 0.05% tween. Following purification samples were concentrated using  
 5 vivaspin 5K cut off concentrators (Vivascience Ltd).

## 2.0 Results

### 2.1 TAR1-5 dimers

6 x 96 clones were picked from the round 2 selection encompassing all the libraries and selection conditions. Supernatant preps were made and assayed by antigen and Protein L  
 10 ELISA, BIAcore and in the receptor assays. In ELISAs, positive binding clones were identified from each selection method and were distributed between 3U, 5U and 7U libraries. However, as the guiding dAb is always present it was not possible to discriminate between high and low affinity binders by this method therefore BIAcore  
 15 analysis was conducted.

BIAcore analysis was conducted using the 2ml supernatants. BIAcore analysis revealed that the dimer Koff rates were vastly improved compared to monomeric TAR1-5. Monomer Koff rate was in the range of  $10^{-1}$ M compared with dimer Koff rates which  
 - 20 were in the range of  $10^{-3}$  -  $10^{-4}$ M. 16 clones that appeared to have very slow off rates were selected, these came from the 3U, 5U and 7U libraries and were sequenced. In addition the supernatants were analysed for the ability to neutralise human TNF $\alpha$  in the receptor assay.

25 6 lead clones (d1-d6 below) that neutralised in these assays and have been sequenced. The results shows that out of the 6 clones obtained there are only 3 different second dAbs (dAb1, dAb2 and dAb3) however where the second dAb is found more than once they are linked with different length linkers.

30 **TAR1-5d1:** 3U linker 2<sup>nd</sup> dAb=dAb1 - 1 $\mu$ g/ml Ag immunotube overnight wash  
**TAR1-5d2:** 3U linker 2<sup>nd</sup> dAb=dAb2 - 1 $\mu$ g/ml Ag immunotube overnight wash  
**TAR1-5d3:** 5U linker 2<sup>nd</sup> dAb=dAb2 - 1 $\mu$ g/ml Ag immunotube overnight wash  
**TAR1-5d4:** 5U linker 2<sup>nd</sup> dAb=dAb3 - 20 $\mu$ g/ml Ag immunotube overnight wash

TAR1-5d5: 5U linker 2<sup>nd</sup> dAb=dAb1 - 20µg/ml Ag immunotube overnight wash

TAR1-5d6: 7U linker 2<sup>nd</sup> dAb=dAb1- R1:1µg/ml Ag immunotube overnight wash,  
R2:beads

- 5 The 6 lead clones were examined further. Protein was produced from the periplasm and supernatant, purified with protein L agarose and examined in the cell and receptor assays. The levels of neutralisation were variable (Table 1). The optimal conditions for protein preparation were determined. Protein produced from HB2151 cells as supernatants gave the highest yield (approximately 10mgs/L of culture). The supernatants were incubated
- 10 with protein L agarose for 2hrs at room temperature or overnight at 4°C. The beads were washed with PBS/NaCl and packed onto an FPLC column using a peristaltic pump. The beads were washed with 10 column volumes of PBS/NaCl and eluted with 0.1M glycine pH2.5. In general, dimeric protein is eluted after the monomer.
- 15 TAR1-5d1-6 dimers were purified by FPLC. Three species were obtained, by FPLC purification and were identified by SDS PAGE. One species corresponds to monomer and the other two species corresponds to dimers of different sizes. The larger of the two species is possibly due to the presence of C terminal tags. These proteins were examined in the receptor assay. The data presented in table 1 represents the optimum results
- 20 obtained from the two dimeric species (Figure 11)

The three second dAbs from the dimer pairs (ie, dAb1, dAb2 and dAb3) were cloned as monomers and examined by ELISA and in the cell and receptor assay. All three dAbs bind specifically to TNF by antigen ELISA and do not cross react with plastic or BSA. As

25 monomers, none of the dAbs neutralise in the cell or receptor assays.

### 2.1.2 TAR1-5-19 dimers

TAR1-5-19 was substituted for TAR1-5 in the 6 lead clones. Analysis of all TAR1-5-19 dimers in the cell and receptor assays was conducted using total protein (protein L

30 purified only) unless otherwise stated (Table 2). TAR1-5-19d4 and TAR1-5-19d3 have the best ND<sub>50</sub> (~5nM) in the cell assay, this is consistent with the receptor assay results and is an improvement over TAR1-5-19 monomer (ND<sub>50</sub>~30nM). Although purified TAR1-5 dimers give variable results in the receptor and cell assays TAR1-5-19 dimers

were more consistent. Variability was shown when using different elution buffers during the protein purification. Elution using 0.1M Phosphate-citrate buffer pH2.6 or 0.2M Glycine pH2.5 although removing all protein from the protein L agarose in most cases rendered it less functional.

5

TAR1-5-19d4 was expressed in the fermenter and purified on cation exchange FPLC to yield a completely pure dimer. As with TAR1-5d4 three species were obtained, by FPLC purification corresponding to monomer and two dimer species. This dimer was amino acid sequenced. TAR1-5-19 monomer and TAR1-5-19d4 were then examined in the  
10 receptor assay and the resulting IC<sub>50</sub> for monomer was 30nM and for dimer was 8nM. The results of the receptor assay comparing TAR1-5-19 monomer, TAR1-5-19d4 and TAR1-5d4 is shown in figure 10.

TAR1-5-19 homodimers were made in the 3U, 5U and 7U vectors, expressed and purified  
15 on Protein L. The proteins were examined in the cell and receptor assays and the resulting IC<sub>50s</sub> (for receptor assay) and ND<sub>50s</sub> (for cell assay) were determined (table 3, figure 12).

## 2.2 Fabs

TAR1-5 and TAR1-5-19 dimers were also cloned into Fab format, expressed and purified  
20 on protein L agarose. Fabs were assessed in the receptor assays (Table 4). The results showed that for both TAR1-5-19 and TAR1-5 dimers the neutralisation levels were similar to the original Gly<sub>4</sub>Ser linker dimers from which they were derived. A TAR1-5-19 Fab where TAR1-5-19 was displayed on both CH and CK was expressed, protein L purified and assessed in the receptor assay. The resulting IC<sub>50</sub> was approximately 1nM.

25

## 2.3 TAR1-27 dimers

3 x 96 clones were picked from the round 2 selection encompassing all the libraries and selection conditions. 2ml supernatant preps were made for analysis in ELISA and  
30 bioassays. Antigen ELISA gave 71 positive clones. The receptor assay of crude supernatants yielded 42 clones with inhibitory properties (TNF binding 0-60%). In the majority of cases inhibitory properties correlated with a strong ELISA signal. 42 clones



were sequenced, 39 of these have unique second dAb sequences. The 12 dimers that gave the best inhibitory properties were analysed further.

The 12 neutralising clones were expressed as 200ml supernatant preps and purified on protein L. These were assessed by protein L and antigen ELISA, BIAcore and in the receptor assay. Strong positive ELISA signals were obtained in all cases. BIAcore analysis revealed all clones to have fast on and off rates. The off rates were improved compared to monomeric TAR1-27, however the off rate of TAR1-27 dimers was faster (Koff is approximately in the range of  $10^{-1}$  and  $10^{-2}$ M) than the TAR1-5 dimers examined previously (Koff is approximately in the range of  $10^{-3}$  -  $10^{-4}$ M). The stability of the purified dimers was questioned and therefore in order to improve stability, the addition on 5%glycerol, 0.5% Triton X100 or 0.5% NP40 (Sigma) was included in the purification of 2 TAR1-27 dimers (d2 and d16). Addition of NP40 or Triton X100™ improved the yield of purified product approximately 2 fold. Both dimers were assessed in the receptor assay. TAR1-27d2 gave IC50 of ~30nM under all purification conditions. TAR1-27d16 showed no neutralisation effect when purified without the use of stabilising agents but gave an IC50 of ~50nM when purified under stabilising conditions. No further analysis was conducted.

#### 2.4 TAR2-5 dimers

3 x 96 clones were picked from the second round selections encompassing all the libraries and selection conditions. 2ml supernatant preps were made for analysis. Protein A and antigen ELISAs were conducted for each plate. 30 interesting clones were identified as having good off-rates by BIAcore (Koff ranges between  $10^{-2}$  –  $10^{-3}$ M). The clones were sequenced and 13 unique dimers were identified by sequence analysis.

**Table 1: TAR1-5 dimers**

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Receptor/ Cell assay
TAR1-5d1	HB2151	Protein L + FPLC	small dimeric species	0.1M glycine pH2.5	RA~30nM
TAR1-5d2	HB2151	Protein L + FPLC	small dimeric species	0.1M glycine pH2.5	RA~50nM

		FPLC	species	pH2.5	M
TAR1-5d3	HB2151	Protein L + FPLC	large dimeric species	0.1M glycine pH2.5	RA~300 nM
TAR1-5d4	HB2151	Protein L + FPLC	small dimeric species	0.1M glycine pH2.5	RA~3n M
TAR1-5d5	HB2151	Protein L + FPLC	large dimeric species	0.1M glycine pH2.5	RA~200 nM
TAR1-5d6	HB2151	Protein L +FPLC	Large dimeric species	0.1M glycine pH2.5	RA~100 nM

\*note dimer 2 and dimer 3 have the same second dAb (called dAb2), however have different linker lengths (d2 = (Gly<sub>4</sub>Ser)<sub>3</sub>, d3 = (Gly<sub>4</sub>Ser)<sub>3</sub>). dAb1 is the partner dAb to dimers 1, 5 and 6. dAb3 is the partner dAb to dimer4. None of the partner dAbs neutralise alone. FPLC purification is by cation exchange unless otherwise stated. The optimal dimeric species for each dimer obtained by FPLC was determined in these assays.

10 **Table 2: TAR1-5-19 dimers**

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Recept or/ Cell assay
TAR1-5-19 d1	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.0	RA~15 nM
TAR1-5-19 d2 (no stop codon)	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.0 + 0.05%NP40	RA~2n M
TAR1-5-19d3 (no stop codon)	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.5 + 0.05%NP40	RA~8n M
TAR1-5-19d4	TOP10F'	Protein L + FPLC	FPLC purified fraction	0.1M glycine pH2.0	RA~2- 5nM CA~12 nM
TAR1-5-19d5	TOP10F'	Protein L	Total protein	0.1M glycine pH2.0 + NP40	RA~8n M CA~10 nM
TAR1-5-19 d6	TOP10F'	Protein L	Total protein	0.1M glycine pH 2.0	RA~10 nM

**Table 3: TAR1-5-19 homodimers**

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Recept or/ Cell assay
-------	-----------	--------------	------------------	--------------------	-----------------------

homodimer						nM CA~30 nM
TAR1-5-19 homodimer	5U	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~2n M CA~3n M
TAR1-5-19 homodimer	7U	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~10 nM CA~15 nM
TAR1-5-19 hinge	cys	HB2151	Protein L + FPLC	FPLC purified dimer fraction	0.1M glycine pH2.5	RA~2n M
TAR1-5-19CH/ TAR1-5-19 CK		HB2151	Protein	Total protein	0.1M glycine pH2.5	RA~1n M

Table 4: TAR1-5/TAR1-5-19 Fabs

Dimer	Cell type	Purification	Protein Fraction	Elution conditions	Rece ptor/ Cell assay
TAR1-5CH/ dAb1 CK	HB2151	Protein L	Total protein	0.1M citrate pH2.6	RA~90 nM
TAR1-5CH/ dAb2 CK	HB2151	Protein L	Total protein	0.1M glycine pH2.5	RA~30 nM CA~60 nM
dAb3CH/ TAR1-5CK	HB2151	Protein L	Total protein	0.1M citrate pH2.6	RA~10 0nM
TAR1-5-19CH/ dAb1 CK	HB2151	Protein L	Total protein	0.1M glycine pH2.0	RA~6n M
dAb1 CH/ TAR1-5-19CK	HB2151	Protein L	0.1M glycine pH2.0	Myc/flag	RA~6n M
TAR1-5-19CH/ dAb2 CK	HB2151	Protein L	Total protein	0.1M glycine pH2.0	RA~8n M CA~12 nM
TAR1-5-19CH/ dAb3CK	HB2151	Protein L	Total protein	0.1M glycine pH2.0	RA~3n M

**PCR construction of TAR1-5-19CYS dimer**

See example 8 describing the dAb trimer. The trimer protocol gives rise to a mixture of monomer, dimer and trimer.

**5 Expression and purification of TAR1-5-19CYS dimer**

The dimer was purified from the supernatant of the culture by capture on Protein L agarose as outlined in the example 8.

**Separation of TAR1-5-19CYS monomer from the TAR1-5-19CYS dimer**

- 10 Prior to cation exchange separation, the mixed monomer/dimer sample was buffer exchanged into 50 mM sodium acetate buffer pH 4.0 using a PD-10 column (Amersham Pharmacia), following the manufacturer's guidelines. The sample was then applied to a 1mL Resource S cation exchange column (Amersham Pharmacia), which had been pre-equilibrated with 50 mM sodium acetate pH 4.0. The monomer and dimer were separated  
15 using the following salt gradient in 50 mM sodium acetate pH 4.0:

150 to 200 mM sodium chloride over 15 column volumes

200 to 450 mM sodium chloride over 10 column volumes

450 to 1000 mM sodium chloride over 15 column volumes

20

Fractions containing dimer only were identified using SDS-PAGE and then pooled and the pH increased to 8 by the addition of 1/5 volume of 1M Tris pH 8.0.

***In vitro* functional binding assay: TNF receptor assay and cell assay**

- 25 The affinity of the dimer for human TNF $\alpha$  was determined using the TNF receptor and cell assay. IC<sub>50</sub> in the receptor assay was approximately 0.3-0.8 nM; ND<sub>50</sub> in the cell assay was approximately 3-8 nM.

**Other possible TAR1-5-19CYS dimer formats**

30

***PEG dimers and custom synthetic maleimide dimers***

Nektar (Shearwater) offer a range of bi-maleimide PEGs [mPEG2-(MAL)<sub>2</sub> or mPEG-(MAL)<sub>2</sub>] which would allow the monomer to be formatted as a dimer, with a small linker

separating the dAbs and both being linked to a PEG ranging in size from 5 to 40 kDa. It has been shown that the 5kDa mPEG-(MAL)<sub>2</sub> (ie, [TAR1-5-19]-Cys-maleimide-PEG x 2, wherein the maleimides are linked together in the dimer) has an affinity in the TNF receptor assay of ~ 1-3 nM. Also the dimer can also be produced using TMEA (Tris[2-maleimidoethyl]amine) (Pierce Biotechnology) or other bi-functional linkers.

It is also possible to produce the disulphide dimer using a chemical coupling procedure using 2,2'-dithiodipyridine (Sigma Aldrich) and the reduced monomer.

#### 10 *Addition of a polypeptide linker or hinge to the C-terminus of the dAb.*

A small linker, either (Gly<sub>4</sub>Ser)<sub>n</sub> where n=1 to 10, eg, 1, 2, 3, 4, 5, 6 or 7, an immunoglobulin (eg, IgG hinge region or random peptide sequence (eg, selected from a library of random peptide sequences) can be engineered between the dAb and the terminal cysteine residue. This can then be used to make dimers as outlined above.

15

### **Example 8**

#### **dAb trimerisation**

20

#### **Summary**

For dAb trimerisation, a free cysteine is required at the C-terminus of the protein. The cysteine residue, once reduced to give the free thiol, can then be used to specifically couple the protein to a trimeric maleimide molecule, for example TMEA (Tris[2-maleimidoethyl]amine).

25

#### **PCR construction of TAR1-5-19CYS**

The following oligonucleotides were used to specifically PCR TAR1-5-19 with a *SalI* and *Bam*HI sites for cloning and also to introduce a C-terminal cysteine residue:

30

*SalI*

-----

35 1 Trp Ser Ala Ser Thr Asp\* Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val  
TGG AGC GCG TCG ACG GAC ATC CAG ATG ACC CAG TCT CCA TCC TCT CTG TCT GCA TCT GTA  
ACC TCG CGC AGC TGC CTG TAG GTC TAC TGG GTC AGA GGT AGG AGA GAC AGA CGT AGA CAT

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Asp Ser Tyr Leu His Trp

109

61 GGA GAC CGT GTC ACC ATC ACT TGC CGG GCA AGT CAG AGC ATT GAT AGT TAT TTA CAT TGG  
 CCT CTG GCA CAG TGG TAG TGA ACG GCC CGT TCA GTC TCG TAA CTA TCA ATA AAT GTA ACC  
  
 Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Glu Leu Gln  
 5 121 TAC CAG CAG AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC TAT AGT GCA TCC GAG TTG CAA  
 ATG GTC GTC TTT GGT CCC TTT CGG GGA TTC GAG GAC TAG ATA TCA CGT AGG CTC AAC GTT  
  
 Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile  
 181 AGT GGG GTC CCA TCA CGT TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC  
 10 TCA CCC CAG GGT AGT GCA AAG TCA CCG TCA CCT AGA CCC TGT CTA AAG TGA GAG TGG TAG  
  
 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Val Val Trp Arg Pro  
 241 AGC AGT CTG CAA CCT GAA GAT TTT GCT ACG TAC TAC TGT CAA CAG GTT GTG TGG CGT CCT  
 TCG TCA GAC GTT GGA CTT CTA AAA CGA TGC ATG ATG ACA GTT GTC CAA CAC ACC GCA GGA  
 15 BamHI  
 -----  
 Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Cys \*\*\* \*\*\* Gly Ser Gly  
 301 TTT ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGG TGC TAA TAA GGA TCC GGC  
 AAA TGC AAG CCG GTT CCC TGG TTC CAC CTT TAG TTT GCC ACG ATT ATT CCT AGG CCG  
 20

(\* start of TAR1-5-19CYS sequence)

Forward primer

25 5'-TGGAGCGCGTCGACGGACATCCAGATGACCCAGTCTCCA-3'

Reverse primer

5'-TTAGCAGCCGGATCCTTATTAGCACCGTTTGATTTCAC-3'

30

The PCR reaction (50µL volume) was set up as follows: 200µM dNTPs, 0.4µM of each  
 primer, 5 µL of 10x *Pfu*Turbo buffer (Stratagene), 100 ng of template plasmid (encoding  
 TAR1-5-19), 1µL of *Pfu*Turbo enzyme (Stratagene) and the volume adjusted to 50µL  
 using sterile water. The following PCR conditions were used: initial denaturing step 94  
 35 °C for 2 mins, then 25 cycles of 94 °C for 30 secs, 64 °C for 30 sec and 72 °C for 30 sec.  
 A final extension step was also included of 72 °C for 5 mins. The PCR product was  
 purified and digested with *Sa*II and *Bam*HI and ligated into the vector which had also  
 been cut with the same restriction enzymes. Correct clones were verified by DNA  
 sequencing.

40

**Expression and purification of TAR1-5-19CYS**

TAR1-5-19CYS vector was transformed into BL21 (DE3) pLysS chemically competent cells (Novagen) following the manufacturer's protocol. Cells carrying the dAb plasmid were selected for using 100 µg/mL carbenicillin and 37 µg/mL chloramphenicol. Cultures were set up in 2L baffled flasks containing 500 mL of terrific broth (Sigma-Aldrich), 100 µg/mL carbenicillin and 37 µg/mL chloramphenicol. The cultures were grown at 30 °C at 200rpm to an O.D.600 of 1-1.5 and then induced with 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside, from Melford Laboratories). The expression of the dAb was allowed to continue for 12-16 hrs at 30 °C. It was found that most of the dAb was present in the culture media. Therefore, the cells were separated from the media by centrifugation (8,000xg for 30 mins), and the supernatant used to purify the dAb. Per litre of supernatant, 30 mL of Protein L agarose (Affitech) was added and the dAb allowed to batch bind with stirring for 2 hours. The resin was then allowed to settle under gravity for a further hour before the supernatant was siphoned off. The agarose was then packed into a XK 50 column (Amersham Pharmacia) and was washed with 10 column volumes of PBS. The bound dAb was eluted with 100 mM glycine pH 2.0 and protein containing fractions were then neutralized by the addition of 1/5 volume of 1 M Tris pH 8.0. Per litre of culture supernatant 20 mg of pure protein was isolated, which contained a 50:50 ratio of monomer to dimer.

20

**Trimerisation of TAR1-5-19CYS**

2.5 ml of 100 µM TAR1-5-19CYS was reduce with 5 mM dithiothreitol and left at room temperature for 20 minutes. The sample was then buffer exchanged using a PD-10 column (Amersham Pharmacia). The column had been pre-equilibrated with 5 mM EDTA, 50 mM sodium phosphate pH 6.5, and the sample applied and eluted following the manufactures guidelines. The sample was placed on ice until required. TMEA (Tris[2-maleimidoethyl]amine) was purchased from Pierce Biotechnology. A 20 mM stock solution of TMEA was made in 100% DMSO (dimethyl sulphoxide). It was found that a concentration of TMEA greater than 3:1 (molar ratio of dAb:TMEA) caused the rapid precipitation and cross-linking of the protein. Also the rate of precipitation and cross-linking was greater as the pH increased. Therefore using 100 µM reduced TAR1-5-19CYS, 25 µM TMEA was added to trimerise the protein and the reaction allowed to proceed at room temperature for two hours. It was found that the addition of additives

30

such as glycerol or ethylene glycol to 20% (v/v), significantly reduced the precipitation of the trimer as the coupling reaction proceeded. After coupling, SDS-PAGE analysis showed the presence of monomer, dimer and trimer in solution.

#### 5 **Purification of the trimeric TAR1-5-19CYS**

40  $\mu$ L of 40% glacial acetic acid was added per mL of the TMEA-TAR1-5-19cys reaction to reduce the pH to ~4. The sample was then applied to a 1mL Resource S cation exchange column (Amersham Pharmacia), which had been pre-equilibrated with 50 mM sodium acetate pH 4.0. The dimer and trimer were partially separated using a salt gradient  
10 of 340 to 450 mM Sodium chloride, 50 mM sodium acetate pH 4.0 over 30 column volumes. Fractions containing trimer only were identified using SDS-PAGE and then pooled and the pH increased to 8 by the addition of 1/5 volume of 1M Tris pH 8.0. To prevent precipitation of the trimer during concentration steps (using 5K cut off Viva spin concentrators; Vivascience), 10% glycerol was added to the sample.

15

#### ***In vitro* functional binding assay: TNF receptor assay and cell assay**

The affinity of the trimer for human TNF $\alpha$  was determined using the TNF receptor and cell assay. IC<sub>50</sub> in the receptor assay was 0.3nM; ND<sub>50</sub> in the cell assay was in the range of 3 to 10nM (eg, 3nM).

20

#### **Other possible TAR1-5-19CYS trimer formats**

TAR1-5-19CYS may also be formatted into a trimer using the following reagents:

##### ***PEG trimers and custom synthetic maleimide trimers***

25 Nektar (Shearwater) offer a range of multi arm PEGs, which can be chemically modified at the terminal end of the PEG. Therefore using a PEG trimer with a maleimide functional group at the end of each arm would allow the trimerisation of the dAb in a manner similar to that outlined above using TMEA. The PEG may also have the advantage in increasing the solubility of the trimer thus preventing the problem of aggregation. Thus, one could  
30 produce a dAb trimer in which each dAb has a C-terminal cysteine that is linked to a maleimide functional group, the maleimide functional groups being linked to a PEG trimer.



*Addition of a polypeptide linker or hinge to the C-terminus of the dAb*

A small linker, either (Gly<sub>4</sub>Ser)<sub>n</sub> where n= 1 to 10, eg, 1, 2, 3, 4, 5, 6 or 7, an immunoglobulin (eg, IgG hinge region or random peptide sequence (eg, selected from a library of random peptide sequences) could be engineered between the dAb and the terminal cysteine residue. When used to make multimers (eg, dimers or trimers), this again would introduce a greater degree of flexibility and distance between the individual monomers, which may improve the binding characteristics to the target, eg a multisubunit target such as human TNF $\alpha$ .

10 **Example 9.****Selection of a collection of single domain antibodies (dAbs) directed against human serum albumin (HSA) and mouse serum albumin (MSA).**

15 This example explains a method for making a single domain antibody (dAb) directed against serum albumin. Selection of dAbs against both mouse serum albumin (MSA) and human serum albumin (HSA) is described. Three human phage display antibody libraries were used in this experiment, each based on a single human framework for V<sub>H</sub> (see Figure 13: sequence of dummy V<sub>H</sub> based on V3-23/DP47 and JH4b) or V<sub>K</sub> (see Figure 20 15: sequence of dummy V<sub>K</sub> based on o12/o2/DPK9 and Jk1) with side chain diversity encoded by NNK codons incorporated in complementarity determining regions (CDR1, CDR2 and CDR3).

Library 1 (V<sub>H</sub>):

25 Diversity at positions: H30, H31, H33, H35, H50, H52, H52a, H53, H55, H56, H58, H95, H97, H98.

Library size:  $6.2 \times 10^9$

Library 2 (V<sub>H</sub>):

30 Diversity at positions: H30, H31, H33, H35, H50, H52, H52a, H53, H55, H56, H58, H95, H97, H98, H99, H100, H100a, H100b.

Library size:  $4.3 \times 10^9$

Library 3 (V $\kappa$ ):

Diversity at positions: L30, L31, L32, L34, L50, L53, L91, L92, L93, L94, L96

Library size:  $2 \times 10^9$

- 5 The V<sub>H</sub> and V $\kappa$  libraries have been preselected for binding to generic ligands protein A and protein L respectively so that the majority of clones in the unselected libraries are functional. The sizes of the libraries shown above correspond to the sizes after preselection.
- 10 Two rounds of selection were performed on serum albumin using each of the libraries separately. For each selection, antigen was coated on immunotube (nunc) in 4ml of PBS at a concentration of 100 $\mu$ g/ml. In the first round of selection, each of the three libraries was panned separately against HSA (Sigma) and MSA (Sigma). In the second round of selection, phage from each of the six first round selections was panned against (i) the
- 15 same antigen again (eg 1<sup>st</sup> round MSA, 2<sup>nd</sup> round MSA) and (ii) against the reciprocal antigen (eg 1<sup>st</sup> round MSA, 2<sup>nd</sup> round HSA) resulting in a total of twelve 2<sup>nd</sup> round selections. In each case, after the second round of selection 48 clones were tested for binding to HSA and MSA. Soluble dAb fragments were produced as described for scFv fragments by Harrison *et al*, Methods Enzymol. 1996;267:83-109 and standard ELISA
- 20 protocol was followed (Hoogenboom *et al*. (1991) Nucleic Acids Res., 19: 4133) except that 2% tween PBS was used as a blocking buffer and bound dAbs were detected with either protein L-HRP (Sigma) (for the V $\kappa$ s) and protein A -HRP (Amersham Pharmacia Biotech) (for the V<sub>H</sub>s).
- 25 dAbs that gave a signal above background indicating binding to MSA, HSA or both were tested in ELISA insoluble form for binding to plastic alone but all were specific for serum albumin. Clones were then sequenced (see table below) revealing that 21 unique dAb sequences had been identified. The minimum similarity (at the amino acid level) between the V $\kappa$  dAb clones selected was 86.25% ((69/80)x100; the result when all the diversified
- 30 residues are different, eg clones 24 and 34). The minimum similarity between the V<sub>H</sub> dAb clones selected was 94 % ((127/136)x100).

Next, the serum albumin binding dAbs were tested for their ability to capture biotinylated antigen from solution. ELISA protocol (as above) was followed except that ELISA plate was coated with 1 µg/ml protein L (for the V<sub>κ</sub> clones) and 1 µg/ml protein A (for the V<sub>H</sub> clones). Soluble dAb was captured from solution as in the protocol and  
 5 detection was with biotinylated MSA or HSA and streptavidin HRP. The biotinylated MSA and HSA had been prepared according to the manufacturer's instructions, with the aim of achieving an average of 2 biotins per serum albumin molecule. Twenty four clones were identified that captured biotinylated MSA from solution in the ELISA. Two of these (clones 2 and 38 below) also captured biotinylated HSA. Next, the dAbs were  
 10 tested for their ability to bind MSA coated on a CM5 biacore chip. Eight clones were found that bound MSA on the biacore.

dAb (all capture biotinylated MSA) V <sub>κ</sub> library 3 template (dummy)	H or κ	CDR1	CDR2	CDR3	Binds MSA in biacore?	Captures biotinylated HSA?
2, 4, 7, 41, 38, 54	κ	SSYLN	RASPLQS	QQTYSVPPT		✓ all 4 bind
46, 47, 52, 56	κ	FKSLK	NASYLQS	QQVVYWPVT		✓ both bind
13, 15	κ	YYHLK	KASTLQS	QQVRKVPRT		
30, 35	κ	RRYLK	QASVLQS	QQGLYPPIT		
19,	κ	YNWLK	RASSLQS	QQNVVIPRT		
22,	κ	LWHLR	HASLLQS	QQSAVYPKT		
23,	κ	FRYLA	HASHLQS	QQRLLYPKT		
24,	κ	FYHLA	PASKLQS	QQRARWPRT		
31,	κ	IWHLN	RASRLQS	QQVARVPRT		
33,	κ	YRYLR	KASSLQS	QQVGYPRT		
34,	κ	LKYLK	NASHLQS	QQTTYYPIT		
53,	κ	LRYLR	KASWLQS	QQVLYYPQT		
11,	κ	LRSLK	AASRLQS	QQVVYWPAT	✓	
12,	κ	FRHLK	AASRLQS	QQVALYPKT	✓	
17,	κ	RKYLR	TASSLQS	QQNLFWPRT	✓	
18,	κ	RRYLN	AASSLQS	QQMLFYYPKT	✓	
16, 21	κ	IKHLK	GASRLQS	QQGARWPQT	✓	
25, 26	κ	YYHLK	KASTLQS	QQVRKVPRT	✓	
27,	κ	YKHLK	NASHLQS	QQVGRYPKT	✓	
55,	κ	FKSLK	NASYLQS	QQVVYWPVT	✓	
V <sub>H</sub> library 1 (and 2) template (dummy)	H	XXYXXX	XIXXXGXXTXYADSVKG	XXXX (XXXX) FDY		
8, 10	H	WVYQMD	SISAFGAKTLYADSVKG	LSGKFDY		
36,	H	WSYQMT	SISSFGSSTLYADSVKG	GRDENYSLFDY		

In all cases the frameworks were identical to the frameworks in the corresponding dummy sequence, with diversity in the CDRs as indicated in the table above.

Of the eight clones that bound MSA on the biacore, two clones that are highly expressed  
5 in *E. coli* (clones MSA16 and MSA26) were chosen for further study (see example 10).  
Full nucleotide and amino acid sequences for MSA16 and 26 are given in figure 16.

#### Example 10.

#### 10 Determination of affinity and serum half-life in mouse of MSA binding dAbs MSA16 and MSA26.

dAbs MSA16 and MSA26 were expressed in the periplasm of *E. coli* and purified using  
batch absorbption to protein L-agarose affinity resin (Affitech, Norway) followed by  
15 elution with glycine at pH 2.2. The purified dAbs were then analysed by inhibition  
biacore to determine  $K_d$ . Briefly, purified MSA16 and MSA26 were tested to determine  
the concentration of dAb required to achieve 200RUs of response on a biacore CM5 chip  
coated with a high density of MSA. Once the required concentrations of dAb had been  
determined, MSA antigen at a range of concentrations around the expected  $K_d$  was  
20 premixed with the dAb and incubated overnight. Binding to the MSA coated biacore chip  
of dAb in each of the premixes was then measured at a high flow-rate of 30  $\mu$ l/minute.  
The resulting curves were used to create Klotz plots, which gave an estimated  $K_d$  of  
200nM for MSA16 and 70nM for MSA 26 (Figure 17 A & B).

25 Next, clones MSA16 and MSA26 were cloned into an expression vector with the HA tag  
(nucleic acid sequence: TATCCTTATGATGTTCTGATTATGCA and amino acid  
sequence: YPYDVPDYA) and 2-10 mg quantities were expressed in *E. coli* and purified  
from the supernatant with protein L-agarose affinity resin (Affitech, Norway) and eluted  
with glycine at pH2.2. Serum half life of the dAbs was determined in mouse. MSA26  
30 and MSA16 were dosed as single i.v. injections at approx 1.5mg/kg into CD1 mice.  
Analysis of serum levels was by goat anti-HA (Abcam, UK) capture and protein L-HRP  
(invitrogen) detection ELISA which was blocked with 4% Marvel. Washing was with  
0.05% tween PBS. Standard curves of known concentrations of dAb were set up in the

presence of 1xmouse serum to ensure comparability with the test samples. Modelling with a 2 compartment model showed MSA-26 had a  $t_{1/2\alpha}$  of 0.16hr, a  $t_{1/2\beta}$  of 14.5hr and an area under the curve (AUC) of 465hr.mg/ml (data not shown) and MSA-16 had a  $t_{1/2\alpha}$  of 0.98hr, a  $t_{1/2\beta}$  of 36.5hr and an AUC of 913hr.mg/ml (figure 18). Both anti-MSA clones had considerably lengthened half life compared with HEL4 (an anti-hen egg white lysozyme dAb) which had a  $t_{1/2\alpha}$  of 0.06hr, and a  $t_{1/2\beta}$  of 0.34hr.

### Example 11.

#### 10 Creation of $V_H$ - $V_H$ and $V_K$ - $V_K$ dual specific Fab like fragments

This example describes a method for making  $V_H$ - $V_H$  and  $V_K$ - $V_K$  dual specifics as Fab like fragments. Before constructing each of the Fab like fragments described, dAbs that bind to targets of choice were first selected from dAb libraries similar to those described in example 9. A  $V_H$  dAb, HEL4, that binds to hen egg lysozyme (Sigma) was isolated and a second  $V_H$  dAb (TAR2h-5) that binds to TNF $\alpha$  receptor (R and D systems) was also isolated. The sequences of these are given in the sequence listing. A  $V_K$  dAb that binds TNF $\alpha$  (TAR1-5-19) was isolated by selection and affinity maturation and the sequence is also set forth in the sequence listing. A second  $V_K$  dAb (MSA 26) described in example 9 whose sequence is in figure 17B was also used in these experiments.

DNA from expression vectors containing the four dAbs described above was digested with enzymes SalI and NotI to excise the DNA coding for the dAb. A band of the expected size (300-400bp) was purified by running the digest on an agarose gel and excising the band, followed by gel purification using the Qiagen gel purification kit (Qiagen, UK). The DNA coding for the dAbs was then inserted into either the  $C_H$  or  $C_K$  vectors (Figs 8 and 9) as indicated in the table below.

dAb	Target antigen	dAb $V_H$ or dAb $V_K$	Inserted into vector	tag ( C terminal)	Antibiotic resistance
HEL4	Hen egg lysozyme	$V_H$	$C_H$	myc	Chloramphenicol
TAR2-5	TNF receptor	$V_H$	$C_K$	flag	Ampicillin
TAR1-5-19	TNF $\alpha$	$V_K$	$C_H$	myc	Chloramphenicol
MSA 26	Mouse serum albumin	$V_K$	$C_K$	flag	Ampicillin

The  $V_H C_H$  and  $V_H C_K$  constructs were cotransformed into HB2151 cells. Separately, the  $V_K C_H$  and  $V_K C_K$  constructs were cotransformed into HB2151 cells. Cultures of each of the cotransformed cell lines were grown overnight (in 2xTy containing 5% glucose, 10 $\mu$ g/ml chloramphenicol and 100 $\mu$ g/ml ampicillin to maintain antibiotic selection for both  $C_H$  and  $C_K$  plasmids). The overnight cultures were used to inoculate fresh media (2xTy, 10 $\mu$ g/ml chloramphenicol and 100 $\mu$ g/ml ampicillin) and grown to OD 0.7-0.9 before induction by the addition of IPTG to express their  $C_H$  and  $C_K$  constructs. Expressed Fab like fragment was then purified from the periplasm by protein A purification (for the cotransformed  $V_H C_H$  and  $V_H C_K$ ) and MSA affinity resin purification (for the cotransformed  $V_K C_H$  and  $V_K C_K$ ).

#### **$V_H$ - $V_H$ dual specific**

Expression of the  $V_H C_H$  and  $V_H C_K$  dual specific was tested by running the protein on a gel. The gel was blotted and a band the expected size for the Fab fragment could be detected on the Western blot via both the myc tag and the flag tag, indicating that both the  $V_H C_H$  and  $V_H C_K$  parts of the Fab like fragment were present. Next, in order to determine whether the two halves of the dual specific were present in the same Fab-like fragment, an ELISA plate was coated overnight at 4°C with 100  $\mu$ l per well of hen egg lysozyme (HEL) at 3 mg/ml in sodium bicarbonate buffer. The plate was then blocked (as described in example 1) with 2% tween PBS followed by incubation with the  $V_H C_H$  / $V_H C_K$  dual specific Fab like fragment. Detection of binding of the dual specific to the HEL was via the non cognate chain using 9e10 (a monoclonal antibody that binds the myc tag, Roche) and anti mouse IgG-HRP (Amersham Pharmacia Biotech). The signal for the  $V_H C_H$  / $V_H C_K$  dual specific Fab like fragment was 0.154 compared to a background signal of 0.069 for the  $V_H C_K$  chain expressed alone. This demonstrates that the Fab like fragment has binding specificity for target antigen.

#### **$V_K$ - $V_K$ dual specific**

After purifying the cotransformed  $V_K C_H$  and  $V_K C_K$  dual specific Fab like fragment on an MSA affinity resin, the resulting protein was used to probe an ELISA plate coated with 1 $\mu$ g/ml TNF $\alpha$  and an ELISA plate coated with 10 $\mu$ g/ml MSA. As predicted, there was signal above background when detected with protein L-HRP on both ELISA plates (data not shown). This indicated that the fraction of protein able to bind to MSA (and therefore

purified on the MSA affinity column) was also able to bind TNF $\alpha$  in a subsequent ELISA, confirming the dual specificity of the antibody fragment. This fraction of protein was then used for two subsequent experiments. Firstly, an ELISA plate coated with 1 $\mu$ g/ml TNF $\alpha$  was probed with dual specific V $\kappa$  C $_H$  and V $\kappa$  C $\kappa$  Fab like fragment and also with a control TNF $\alpha$  binding dAb at a concentration calculated to give a similar signal on the ELISA. Both the dual specific and control dAb were used to probe the ELISA plate in the presence and in the absence of 2mg/ml MSA. The signal in the dual specific well was reduced by more than 50% but the signal in the dAb well was not reduced at all (see figure 19a). The same protein was also put into the receptor assay with and without MSA and competition by MSA was also shown (see figure 19c). This demonstrates that binding of MSA to the dual specific is competitive with binding to TNF $\alpha$ .

#### Example 12.

##### Creation of a V $\kappa$ - V $\kappa$ dual specific cys bonded dual specific with specificity for mouse serum albumin and TNF $\alpha$

This example describes a method for making a dual specific antibody fragment specific for both mouse serum albumin and TNF $\alpha$  by chemical coupling via a disulphide bond. Both MSA16 (from example 1) and TAR1-5-19 dAbs were recloned into a pET based vector with a C terminal cysteine and no tags. The two dAbs were expressed at 4-10 mg levels and purified from the supernatant using protein L-agarose affinity resin (Affittech, Norway). The cysteine tagged dAbs were then reduced with dithiothreitol. The TAR1-5-19 dAb was then coupled with dithiodipyridine to block reformation of disulphide bonds resulting in the formation of PEP 1-5-19 homodimers. The two different dAbs were then mixed at pH 6.5 to promote disulphide bond formation and the generation of TAR1-5-19, MSA16 cys bonded heterodimers. This method for producing conjugates of two unlike proteins was originally described by King *et al.* (King TP, Li Y Kochoumian L Biochemistry. 1978 vol17:1499-506 Preparation of protein conjugates via intermolecular disulfide bond formation.) Heterodimers were separated from monomeric species by cation exchange. Separation was confirmed by the presence of a band of the expected

size on a SDS gel. The resulting heterodimeric species was tested in the TNF receptor assay and found to have an IC<sub>50</sub> for neutralising TNF of approximately 18 nM. Next, the receptor assay was repeated with a constant concentration of heterodimer (18nM) and a dilution series of MSA and HSA. The presence of HSA at a range of concentrations (up to 2 mg/ml) did not cause a reduction in the ability of the dimer to inhibit TNF $\alpha$ . However, the addition of MSA caused a dose dependant reduction in the ability of the dimer to inhibit TNF $\alpha$  (figure 20). This demonstrates that MSA and TNF $\alpha$  compete for binding to the cys bonded TAR1-5-19, MSA16 dimer.

#### 10 Data Summary

A summary of data obtained in the experiments set forth in the foregoing examples is set forth in Annex 4.

All publications mentioned in the present specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.



**Annex 1; polypeptides which enhance half-life *in vivo*.**

- Alpha-1 Glycoprotein (Orosomucoid) (AAG)
- Alpha-1 Antichyromotrypsin (ACT)
- 5 Alpha-1 Antitrypsin (AAT)
- Alpha-1 Microglobulin (Protein HC) (AIM)
- Alpha-2 Macroglobulin (A2M)
- Antithrombin III (AT III)
- Apolipoprotein A-1 (Apo A-1)
- 10 Apolipoprotein B (Apo B)
- Beta-2-microglobulin (B2M)
- Ceruloplasmin (Cp)
- Complement Component (C3)
- Complement Component (C4)
- 15 C1 Esterase Inhibitor (C1 INH)
- C-Reactive Protein (CRP)
- Cystatin C (Cys C)
- Ferritin (FER)
- Fibrinogen (FIB)
- 20 Fibronectin (FN)
- Haptoglobin (Hp)
- Hemopexin (HPX)
- Immunoglobulin A (IgA)
- Immunoglobulin D (IgD)
- 25 Immunoglobulin E (IgE)
- Immunoglobulin G (IgG)
- Immunoglobulin M (IgM)
- Immunoglobulin Light Chains (kappa/lambda)
- Lipoprotein(a) [Lp(a)]
- 30 Mannose-binding protein (MBP)
- Myoglobin (Myo)
- Plasminogen (PSM)
- Prealbumin (Transthyretin) (PAL)

Retinol-binding protein (RBP)

Rheumatoid Factor (RF)

Serum Amyloid A (SAA)

Soluble Transferrin Receptor (sTfR)

5 Transferrin (Tf)

## Annex 2

Pairing	Therapeutic relevant references.
TNF ALPHA/TGF- $\beta$	<ul style="list-style-type: none"> <li>TGF-<math>\beta</math> and TNF when injected into the ankle joint of collagen induced arthritis model significantly enhanced joint inflammation. In non-collagen challenged mice there was no effect.</li> </ul>
TNF ALPHA/IL-1	<ul style="list-style-type: none"> <li>TNF and IL-1 synergize in the pathology of uveitis.</li> <li>TNF and IL-1 synergize in the pathology of malaria (hypoglycaemia, NO).</li> <li>TNF and IL-1 synergize in the induction of polymorphonuclear (PMN) cells migration in inflammation.</li> <li>IL-1 and TNF synergize to induce PMN infiltration into the peritoneum.</li> <li>IL-1 and TNF synergize to induce the secretion of IL-1 by endothelial cells. Important in inflammation.</li> <li>IL-1 or TNF alone induced some cellular infiltration into knee synovium. IL-1 induced PMNs, TNF – monocytes. Together they induced a more severe infiltration due to increased PMNs.</li> <li>Circulating myocardial depressant substance (present in sepsis) is low levels of IL-1 and TNF acting synergistically.</li> </ul>
TNF ALPHA/IL-2	<ul style="list-style-type: none"> <li>Most relating to synergistic activation of killer T-cells.</li> </ul>
TNF ALPHA/IL-3	<ul style="list-style-type: none"> <li>Synergy of interleukin 3 and tumor necrosis factor alpha in stimulating clonal growth of acute myelogenous leukemia blasts is the result of induction of secondary hematopoietic cytokines by tumor necrosis factor alpha.</li> <li>Cancer Res. 1992 Apr 15;52(8):2197-201.</li> </ul>
TNF ALPHA/IL-4	<ul style="list-style-type: none"> <li>IL-4 and TNF synergize to induce VCAM expression on endothelial cells. Implied to have a role in asthma. Same for synovium – implicated in RA.</li> <li>TNF and IL-4 synergize to induce IL-6 expression in keratinocytes.</li> <li>Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-alpha in combination with either IL-4 or IL-13 through increased mRNA stability. <i>Am J Pathol.</i> 1999 Apr;154(4):1149-58</li> </ul>
TNF ALPHA/IL-5	<ul style="list-style-type: none"> <li>Relationship between the tumor necrosis factor system and the serum interleukin-4, interleukin-5, interleukin-8, eosinophil cationic protein, and immunoglobulin E levels in the bronchial hyperreactivity of adults and their children. <i>Allergy Asthma Proc.</i> 2003 Mar-Apr;24(2):111-8.</li> </ul>
TNF ALPHA/IL-6	<ul style="list-style-type: none"> <li>TNF and IL-6 are potent growth factors for OH-2, a novel human myeloma cell line. <i>Eur J Haematol.</i> 1994 Jul;53(1):31-7.</li> </ul>
TNF ALPHA/IL-8	<ul style="list-style-type: none"> <li>TNF and IL-8 synergized with PMNs to activate platelets. Implicated in Acute Respiratory Distress Syndrome.</li> <li>See IL-5/TNF (asthma). Synergism between interleukin-8 and tumor necrosis factor-alpha for neutrophil-mediated platelet activation. <i>Eur Cytokine Netw.</i> 1994 Sep-Oct;5(5):455-60. (adult respiratory distress syndrome (ARDS))</li> </ul>
TNF ALPHA/IL-9	
TNF ALPHA/IL-10	<ul style="list-style-type: none"> <li>IL-10 induces and synergizes with TNF in the induction of HIV expression in chronically infected T-cells.</li> </ul>
TNF ALPHA/IL-11	<ul style="list-style-type: none"> <li>Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. <i>Am J Physiol Cell Physiol.</i> 2002 Sep;283(3):C679-87. (Bone loss)</li> </ul>
TNF ALPHA/IL-12	

TNF ALPHA/IL-13	<ul style="list-style-type: none"> <li>Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-alpha in combination with either IL-4 or IL-13 through increased mRNA stability. <i>Am J Pathol.</i> 1999 Apr;154(4):1149-58.</li> <li>Interleukin-13 and tumour necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. <i>Clin Exp Allergy.</i> 2000 Mar;30(3):348-55.</li> <li>Interleukin-13 and tumour necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. <i>Clin Exp Allergy.</i> 2000 Mar;30(3):348-55 (allergic inflammation)</li> <li>Implications of serum TNF-beta and IL-13 in the treatment response of childhood nephrotic syndrome. <i>Cytokine.</i> 2003 Feb 7;21(3):155-9.</li> </ul>
TNF ALPHA/IL-14	<ul style="list-style-type: none"> <li>Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma. <i>Thorax.</i> 2002 Sep;57(9):774-8.</li> </ul>
TNF ALPHA/IL-15	<ul style="list-style-type: none"> <li>Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma. <i>Thorax.</i> 2002 Sep;57(9):774-8.</li> </ul>
TNF ALPHA/IL-16	<ul style="list-style-type: none"> <li>Tumor necrosis factor-alpha-induced synthesis of interleukin-16 in airway epithelial cells: priming for serotonin stimulation. <i>Am J Respir Cell Mol Biol.</i> 2003 Mar;28(3):354-62. (airway inflammation)</li> <li>Correlation of circulating interleukin 16 with proinflammatory cytokines in patients with rheumatoid arthritis. <i>Rheumatology</i> (Oxford). 2001 Apr;40(4):474-5. No abstract available.</li> <li>Interleukin 16 is up-regulated in Crohn's disease and participates in TNBS colitis in mice. <i>Gastroenterology.</i> 2000 Oct;119(4):972-82.</li> </ul>
TNF ALPHA/IL-17	<ul style="list-style-type: none"> <li>Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with <i>Borrelia burgdorferi</i>. <i>Infect Immun.</i> 2003 Jun;71(6):3437-42.</li> <li>Interleukin 17 synergises with tumour necrosis factor alpha to induce cartilage destruction <i>in vitro</i>. <i>Ann Rheum Dis.</i> 2002 Oct;61(10):870-6.</li> <li>A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. <i>Eur Respir J.</i> 2003 Mar;21(3):387-93. (Airway inflammation)</li> <li>Abstract Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. <i>Arthritis Rheum.</i> 2001 Sep;44(9):2078-83.</li> </ul>
TNF ALPHA/IL-18	<ul style="list-style-type: none"> <li>Association of interleukin-18 expression with enhanced levels of both interleukin-1beta and tumor necrosis factor alpha in knee synovial tissue of patients with rheumatoid arthritis. <i>Arthritis Rheum.</i> 2003 Feb;48(2):339-47.</li> <li>Abstract Elevated levels of interleukin-18 and tumor necrosis factor-alpha in serum of patients with type 2 diabetes mellitus: relationship with diabetic nephropathy. <i>Metabolism.</i> 2003 May;52(5):605-8.</li> </ul>
TNF ALPHA/IL-19	<ul style="list-style-type: none"> <li>Abstract IL-19 induces production of IL-6 and TNF-alpha and results in cell apoptosis through TNF-alpha. <i>J Immunol.</i> 2002 Oct 15;169(8):4288-97.</li> </ul>
TNF ALPHA/IL-20	<ul style="list-style-type: none"> <li>Abstract Cytokines: IL-20 - a new effector in skin inflammation. <i>Curr Biol.</i> 2001 Jul 10;11(13):R531-4</li> </ul>
TNF ALPHA/Complement	<ul style="list-style-type: none"> <li>Inflammation and coagulation: implications for the septic patient. <i>Clin Infect Dis.</i> 2003 May 15;36(10):1259-65. Epub 2003 May 08. Review.</li> </ul>
TNF ALPHA/IFN- $\gamma$	<ul style="list-style-type: none"> <li>MHC induction in the brain.</li> <li>Synergize in anti-viral response/IFN-<math>\beta</math> induction.</li> <li>Neutrophil activation/ respiratory burst.</li> <li>Endothelial cell activation</li> <li>Toxicities noted when patients treated with TNF/IFN-<math>\gamma</math> as anti-viral therapy</li> </ul>

	<ul style="list-style-type: none"> <li>• Fractalkine expression by human astrocytes.</li> <li>• Many papers on inflammatory responses – i.e. LPS, also macrophage activation.</li> <li>• Anti-TNF and anti-IFN-<math>\gamma</math> synergize to protect mice from lethal endotoxemia.</li> </ul>
TGF- $\beta$ /IL-1	<ul style="list-style-type: none"> <li>• Prostaglandin synthesis by osteoblasts</li> <li>• IL-6 production by intestinal epithelial cells (inflammation model)</li> <li>• Stimulates IL-11 and IL-6 in lung fibroblasts (inflammation model)</li> <li>• IL-6 and IL-8 production in the retina</li> </ul>
TGF- $\beta$ /IL-6	<ul style="list-style-type: none"> <li>• Chondrocarcoma proliferation</li> </ul>
IL-1/IL-2	<ul style="list-style-type: none"> <li>• B-cell activation</li> <li>• LAK cell activation</li> <li>• T-cell activation</li> <li>• IL-1 synergy with IL-2 in the generation of lymphokine activated killer cells is mediated by TNF-alpha and beta (lymphotoxin). <i>Cytokine</i>. 1992 Nov;4(6):479-87.</li> </ul>
IL-1/IL-3	
IL-1/IL-4	<ul style="list-style-type: none"> <li>• B-cell activation</li> <li>• IL-4 induces IL-1 expression in endothelial cell activation.</li> </ul>
IL-1/IL-5	
IL-1/IL-6	<ul style="list-style-type: none"> <li>• B cell activation</li> <li>• T cell activation (can replace accessory cells)</li> <li>• IL-1 induces IL-6 expression</li> <li>• C3 and serum amyloid expression (acute phase response)</li> <li>• HIV expression</li> <li>• Cartilage collagen breakdown.</li> </ul>
IL-1/IL-7	<ul style="list-style-type: none"> <li>• IL-7 is requisite for IL-1-induced thymocyte proliferation. Involvement of IL-7 in the synergistic effects of granulocyte-macrophage colony-stimulating factor or tumor necrosis factor with IL-1. <i>J Immunol</i>. 1992 Jan 1;148(1):99-105.</li> </ul>
IL-1/IL-8	
IL-1/IL-10	
IL-1/IL-11	<ul style="list-style-type: none"> <li>• Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. <i>Am J Physiol Cell Physiol</i>. 2002 Sep;283(3):C679-87. (Bone loss)</li> </ul>
IL-1/IL-16	<ul style="list-style-type: none"> <li>• Correlation of circulating interleukin 16 with proinflammatory cytokines in patients with rheumatoid arthritis. <i>Rheumatology (Oxford)</i>. 2001 Apr;40(4):474-5. No abstract available.</li> </ul>
IL-1/IL-17	<ul style="list-style-type: none"> <li>• Inhibition of interleukin-17 prevents the development of arthritis in vaccinated mice challenged with <i>Borrelia burgdorferi</i>. <i>Infect Immun</i>. 2003 Jun;71(6):3437-42.</li> <li>• Contribution of interleukin 17 to human cartilage degradation and synovial inflammation in osteoarthritis. <i>Osteoarthritis Cartilage</i>. 2002 Oct;10(10):799-807.</li> <li>• Abstract Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci. <i>Arthritis Rheum</i>. 2001 Sep;44(9):2078-83.</li> </ul>
IL-1/IL-18	<ul style="list-style-type: none"> <li>• Association of interleukin-18 expression with enhanced levels of both interleukin-1beta and tumor necrosis factor alpha in knee synovial tissue of patients with rheumatoid arthritis. <i>Arthritis Rheum</i>. 2003 Feb;48(2):339-47.</li> </ul>
IL-1/IFN- $\gamma$	
IL-2/IL-3	<ul style="list-style-type: none"> <li>• T-cell proliferation</li> <li>• B cell proliferation</li> </ul>

IL-2/IL-4	<ul style="list-style-type: none"> <li>B-cell proliferation</li> <li>T-cell proliferation</li> <li>(selectively inducing activation of CD8 and NK lymphocytes)IL-2R beta agonist P1-30 acts in synergy with IL-2, IL-4, IL-9, and IL-15: biological and molecular effects. <i>J Immunol.</i> 2000 Oct 15;165(8): 4312-8.</li> </ul>
IL-2/IL-5	<ul style="list-style-type: none"> <li>B-cell proliferation/ Ig secretion</li> <li>IL-5 induces IL-2 receptors on B-cells</li> </ul>
IL-2/IL-6	<ul style="list-style-type: none"> <li>Development of cytotoxic T-cells</li> </ul>
IL-2/IL-7	
IL-2/IL-9	<ul style="list-style-type: none"> <li>See IL-2/IL-4 (NK-cells)</li> </ul>
IL-2/IL-10	<ul style="list-style-type: none"> <li>B-cell activation</li> </ul>
IL-2/IL-12	<ul style="list-style-type: none"> <li>IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. <i>Cell Immunol.</i> 1995 Oct 1;165(1):33-43. (T-cell activation)</li> </ul>
IL-2/IL-15	<ul style="list-style-type: none"> <li>See IL-2/IL-4 (NK cells)</li> <li>(T cell activation and proliferation) IL-15 and IL-2: a matter of life and death for T cells <i>in vivo</i>. <i>Nat Med.</i> 2001 Jan;7(1):114-8.</li> </ul>
IL-2/IL-16	<ul style="list-style-type: none"> <li>Synergistic activation of CD4+ T cells by IL-16 and IL-2. <i>J Immunol.</i> 1998 Mar 1;160(5):2115-20.</li> </ul>
IL-2/IL-17	<ul style="list-style-type: none"> <li>Evidence for the early involvement of interleukin 17 in human and experimental renal allograft rejection. <i>J Pathol.</i> 2002 Jul;197(3):322-32.</li> </ul>
IL-2/IL-18	<ul style="list-style-type: none"> <li>Interleukin 18 (IL-18) in synergy with IL-2 induces lethal lung injury in mice: a potential role for cytokines, chemokines, and natural killer cells in the pathogenesis of interstitial pneumonia. <i>Blood.</i> 2002 Feb 15;99(4):1289-98.</li> </ul>
IL-2/TGF- $\beta$	<ul style="list-style-type: none"> <li>Control of CD4 effector fate: transforming growth factor beta 1 and interleukin 2 synergize to prevent apoptosis and promote effector expansion. <i>J Exp Med.</i> 1995 Sep 1;182(3):699-709.</li> </ul>
IL-2/IFN- $\gamma$	<ul style="list-style-type: none"> <li>Ig secretion by B-cells</li> <li>IL-2 induces IFN-<math>\gamma</math> expression by T-cells</li> </ul>
IL-2/IFN- $\alpha/\beta$	<ul style="list-style-type: none"> <li>None</li> </ul>
IL-3/IL-4	<ul style="list-style-type: none"> <li>Synergize in mast cell growth</li> <li>Synergistic effects of IL-4 and either GM-CSF or IL-3 on the induction of CD23 expression by human monocytes: regulatory effects of IFN-alpha and IFN-gamma. <i>Cytokine.</i> 1994 Jul;6(4):407-13.</li> </ul>
IL-3/IL-5	
IL-3/IL-6	
IL-3/IFN- $\gamma$	<ul style="list-style-type: none"> <li>IL-4 and IFN-gamma synergistically increase total polymeric IgA receptor levels in human intestinal epithelial cells. Role of protein tyrosine kinases. <i>J Immunol.</i> 1996 Jun 15;156(12):4807-14.</li> </ul>
IL-3/GM-CSF	<ul style="list-style-type: none"> <li>Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. <i>J Immunol.</i> 2003 Jun 1;170(11):5359-66. (allergic inflammation)</li> </ul>
IL-4/IL-2	<ul style="list-style-type: none"> <li>IL-4 synergistically enhances both IL-2- and IL-12-induced IFN-<math>\gamma</math> expression in murine NK cells. <i>Blood.</i> 2003 Mar 13 [Epub ahead of print]</li> </ul>
IL-4/IL-5	<ul style="list-style-type: none"> <li>Enhanced mast cell histamine etc. secretion in response to IgE</li> <li>A Th2-like cytokine response is involved in bullous pemphigoid. the role of IL-4 and IL-5 in the pathogenesis of the disease. <i>Int J Immunopathol Pharmacol.</i> 1999 May-Aug;12(2):55-61.</li> </ul>
IL-4/IL-6	
IL-4/IL-10	
IL-4/IL-11	<ul style="list-style-type: none"> <li>Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. <i>Blood.</i></li> </ul>

	1991 Sep 15;78(6):1448-51.
IL-4/IL-12	<ul style="list-style-type: none"> <li>• Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-gamma production by dendritic cells. <i>J Immunol.</i> 2000 Jan 1;164(1):64-71. (increase Th1/Th2 differentiation)</li> <li>• IL-4 synergistically enhances both IL-2- and IL-12-induced IFN-<math>\gamma</math> expression in murine NK cells. <i>Blood.</i> 2003 Mar 13 [Epub ahead of print]</li> </ul>
IL-4/IL-13	<ul style="list-style-type: none"> <li>• Abstract Interleukin-4 and interleukin-13 signaling connections maps. <i>Science.</i> 2003 Jun 6;300(5625):1527-8. (allergy, asthma)</li> <li>• Inhibition of the IL-4/IL-13 receptor system prevents allergic sensitization without affecting established allergy in a mouse model for allergic asthma. <i>J Allergy Clin Immunol.</i> 2003 Jun;111(6):1361-1369.</li> </ul>
IL-4/IL-16	<ul style="list-style-type: none"> <li>• (asthma) Interleukin (IL)-4/IL-9 and exogenous IL-16 induce IL-16 production by BEAS-2B cells, a bronchial epithelial cell line. <i>Cell Immunol.</i> 2001 Feb 1;207(2):75-80</li> </ul>
IL-4/IL-17	<ul style="list-style-type: none"> <li>• Interleukin (IL)-4 and IL-17 synergistically stimulate IL-6 secretion in human colonic myofibroblasts. <i>Int J Mol Med.</i> 2002 Nov;10(5):631-4. (Gut inflammation)</li> </ul>
IL-4/IL-24	<ul style="list-style-type: none"> <li>• IL-24 is expressed by rat and human macrophages. <i>Immunobiology.</i> 2002 Jul;205(3):321-34.</li> </ul>
IL-4/IL-25	<ul style="list-style-type: none"> <li>• Abstract New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. <i>J Immunol.</i> 2002 Jul 1;169(1):443-53. (allergic inflammation)</li> <li>• Abstract Mast cells produce interleukin-25 upon Fcepsilon RI-mediated activation. <i>Blood.</i> 2003 May 1;101(9):3594-6. Epub 2003 Jan 02. (allergic inflammation)</li> </ul>
IL-4/IFN- $\gamma$	<ul style="list-style-type: none"> <li>• Abstract Interleukin 4 induces interleukin 6 production by endothelial cells: synergy with interferon-gamma. <i>Eur J Immunol.</i> 1991 Jan;21(1):97-101.</li> </ul>
IL-4/SCF	<ul style="list-style-type: none"> <li>• Regulation of human intestinal mast cells by stem cell factor and IL-4. <i>Immunol Rev.</i> 2001 Feb;179:57-60. Review.</li> </ul>
IL-5/IL-3	<ul style="list-style-type: none"> <li>• Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. <i>J Immunol.</i> 2003 Jun 1;170(11):5359-66. (Allergic inflammation see abstract)</li> </ul>
IL-5/IL-6	
IL-5/IL-13	<ul style="list-style-type: none"> <li>• Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: role of eosinophils, IL-5, eotaxin, and IL-13. <i>J Allergy Clin Immunol.</i> 2003 May;111(5):1049-61.</li> </ul>
IL-5/IL-17	<ul style="list-style-type: none"> <li>• Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. <i>Am J Respir Cell Mol Biol.</i> 2003 Jan;28(1):42-50.</li> </ul>
IL-5/IL-25	<ul style="list-style-type: none"> <li>• Abstract New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. <i>J Immunol.</i> 2002 Jul 1;169(1):443-53. (allergic inflammation)</li> <li>• Abstract Mast cells produce interleukin-25 upon Fcepsilon RI-mediated activation. <i>Blood.</i> 2003 May 1;101(9):3594-6. Epub 2003 Jan 02. (allergic inflammation)</li> </ul>
IL-5/IFN- $\gamma$	
IL-5/GM-CSF	<ul style="list-style-type: none"> <li>• Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. <i>J Immunol.</i> 2003 Jun 1;170(11):5359-66. (Allergic inflammation)</li> </ul>
IL-6/IL-10	
IL-6/IL-11	
IL-6/IL-16	<ul style="list-style-type: none"> <li>• Interleukin-16 stimulates the expression and production of pro-</li> </ul>

	inflammatory cytokines by human monocytes. <i>Immunology</i> . 2000 May;100(1):63-9.
IL-6/IL-17	<ul style="list-style-type: none"> <li>Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. <i>J Biol Chem</i>. 2003 May 9;278(19):17036-43. Epub 2003 Mar 06. (airway inflammation, asthma)</li> </ul>
IL-6/IL-19	<ul style="list-style-type: none"> <li>Abstract IL-19 induces production of IL-6 and TNF-alpha and results in cell apoptosis through TNF-alpha. <i>J Immunol</i>. 2002 Oct 15;169(8):4288-97.</li> </ul>
IL-6/IFN-g	
IL-7/IL-2	<ul style="list-style-type: none"> <li>Interleukin 7 worsens graft-versus-host disease. <i>Blood</i>. 2002 Oct 1;100(7):2642-9.</li> </ul>
IL-7/IL-12	<ul style="list-style-type: none"> <li>Synergistic effects of IL-7 and IL-12 on human T cell activation. <i>J Immunol</i>. 1995 May 15;154(10):5093-102.</li> </ul>
IL-7/IL-15	<ul style="list-style-type: none"> <li>Interleukin-7 and interleukin-15 regulate the expression of the bcl-2 and c-myc genes in cutaneous T-cell lymphoma cells. <i>Blood</i>. 2001 Nov 1;98(9):2778-83. (growth factor)</li> </ul>
IL-8/IL-11	<ul style="list-style-type: none"> <li>Abnormal production of interleukin (IL)-11 and IL-8 in polycythaemia vera. <i>Cytokine</i>. 2002 Nov 21;20(4):178-83.</li> </ul>
IL-8/IL-17	<ul style="list-style-type: none"> <li>The Role of IL-17 in Joint Destruction. <i>Drug News Perspect</i>. 2002 Jan;15(1):17-23. (arthritis)</li> <li>Abstract Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. <i>Am J Respir Cell Mol Biol</i>. 2002 Jun;26(6):748-53. (airway inflammation)</li> </ul>
IL-8/GSF	<ul style="list-style-type: none"> <li>Interleukin-8: an autocrine/paracrine growth factor for human hematopoietic progenitors acting in synergy with colony stimulating factor-1 to promote monocyte-macrophage growth and differentiation. <i>Exp Hematol</i>. 1999 Jan;27(1):28-36.</li> </ul>
IL-8/VEGF	<ul style="list-style-type: none"> <li>Intracavitary VEGF, bFGF, IL-8, IL-12 levels in primary and recurrent malignant glioma. <i>J Neurooncol</i>. 2003 May;62(3):297-303.</li> </ul>
IL-9/IL-4	<ul style="list-style-type: none"> <li>Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. <i>Am J Respir Crit Care Med</i>. 2002 Aug 1;166(3):409-16.</li> </ul>
IL-9/IL-5	<ul style="list-style-type: none"> <li>Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. <i>J Clin Invest</i>. 2002 Jan;109(1):29-39.</li> <li>Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. <i>Respir Res</i>. 2001;2(2):80-4. Epub 2001 Feb 15. Review.</li> <li>Abstract Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. <i>Blood</i>. 2000 Sep 15;96(6):2163-71 (asthma)</li> </ul>
IL-9/IL-13	<ul style="list-style-type: none"> <li>Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. <i>Am J Respir Crit Care Med</i>. 2002 Aug 1;166(3):409-16.</li> <li>Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. <i>Nat Med</i>. 2002 Aug;8(8):885-9.</li> </ul>
IL-9/IL-16	<ul style="list-style-type: none"> <li>See IL-4/IL-16</li> </ul>
IL-10/IL-2	<ul style="list-style-type: none"> <li>The interplay of interleukin-10 (IL-10) and interleukin-2 (IL-2) in humoral immune responses: IL-10 synergizes with IL-2 to enhance responses of human B lymphocytes in a mechanism which is different from upregulation of CD25 expression. <i>Cell Immunol</i>. 1994 Sep;157(2):478-88.</li> </ul>
IL-10/IL-12	
IL-10/TGF-beta	<ul style="list-style-type: none"> <li>IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. <i>Eur J Immunol</i>. 2003 May;33(5):1205-14.</li> </ul>
IL-10/IFN-gamma	



IL-11/IL-6	<ul style="list-style-type: none"> <li>Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. <i>Bone</i>. 2003 Jan;32(1):1-7. (bone resorption in inflammation)</li> </ul>
IL-11/IL-17	<ul style="list-style-type: none"> <li>Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. <i>J Allergy Clin Immunol</i>. 2003 Apr;111(4):875-81. (allergic dermatitis)</li> <li>IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. <i>J Immunol</i>. 2003 Mar 1;170(5):2655-62.</li> </ul>
IL-11/TGF- $\beta$	<ul style="list-style-type: none"> <li>Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. <i>J Allergy Clin Immunol</i>. 2003 Apr;111(4):875-81. (allergic dermatitis)</li> </ul>
IL-12/IL-13	<ul style="list-style-type: none"> <li>Relationship of Interleukin-12 and Interleukin-13 imbalance with class-specific rheumatoid factors and anticardiolipin antibodies in systemic lupus erythematosus. <i>Clin Rheumatol</i>. 2003 May;22(2):107-11.</li> </ul>
IL-12/IL-17	<ul style="list-style-type: none"> <li>Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. <i>Scand J Gastroenterol</i>. 2003 Feb;38(2):180-5.</li> </ul>
IL-12/IL-18	<ul style="list-style-type: none"> <li>Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. <i>Cytokine</i>. 1999 Nov;11(11):822-30.</li> <li>Inflammatory Liver Steatosis Caused by IL-12 and IL-18. <i>J Interferon Cytokine Res</i>. 2003 Mar;23(3):155-62.</li> </ul>
IL-12/IL-23	<ul style="list-style-type: none"> <li>Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. <i>Nature</i>. 2003 Feb 13;421(6924):744-8.</li> <li>Abstract A unique role for IL-23 in promoting cellular immunity. <i>J Leukoc Biol</i>. 2003 Jan;73(1):49-56. Review.</li> </ul>
IL-12/IL-27	<ul style="list-style-type: none"> <li>Abstract IL-27, a heterodimeric cytokine composed of EB13 and p28 protein, induces proliferation of naive CD4(+) T cells. <i>Immunity</i>. 2002 Jun;16(6):779-90.</li> </ul>
IL-12/IFN- $\gamma$	<ul style="list-style-type: none"> <li>IL-12 induces IFN-<math>\gamma</math> expression by B and T-cells as part of immune stimulation.</li> </ul>
IL-13/IL-5	<ul style="list-style-type: none"> <li>See IL-5/IL-13</li> </ul>
IL-13/IL-25	<ul style="list-style-type: none"> <li>Abstract New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. <i>J Immunol</i>. 2002 Jul 1;169(1):443-53. (allergic inflammation)</li> <li>Abstract Mast cells produce interleukin-25 upon Fcepsilon RI-mediated activation. <i>Blood</i>. 2003 May 1;101(9):3594-6. Epub 2003 Jan 02. (allergic inflammation)</li> </ul>
IL-15/IL-13	<ul style="list-style-type: none"> <li>Differential expression of interleukins (IL)-13 and IL-15 in ectopic and eutopic endometrium of women with endometriosis and normal fertile women. <i>Am J Reprod Immunol</i>. 2003 Feb;49(2):75-83.</li> </ul>
IL-15/IL-16	<ul style="list-style-type: none"> <li>IL-15 and IL-16 overexpression in cutaneous T-cell lymphomas: stage-dependent increase in mycosis fungoides progression. <i>Exp Dermatol</i>. 2000 Aug;9(4):248-51.</li> </ul>
IL-15/IL-17	<ul style="list-style-type: none"> <li>Abstract IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. <i>J Immunol</i>. 2003 Feb 15;170(4):2106-12. (airway inflammation)</li> </ul>
IL-15/IL-21	<ul style="list-style-type: none"> <li>IL-21 in Synergy with IL-15 or IL-18 Enhances IFN-gamma Production in Human NK and T Cells. <i>J Immunol</i>. 2003 Jun 1;170(11):5464-9.</li> </ul>
IL-17/IL-23	<ul style="list-style-type: none"> <li>Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. <i>J Biol Chem</i>. 2003 Jan 17;278(3):1910-4. Epub 2002 Nov 03</li> </ul>
IL-17/TGF- $\beta$	<ul style="list-style-type: none"> <li>Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. <i>J Allergy Clin Immunol</i>. 2003 Apr;111(4):875-81. (allergic dermatitis)</li> </ul>
IL-18/IL-12	<ul style="list-style-type: none"> <li>Synergistic proliferation and activation of natural killer cells by interleukin</li> </ul>

	<p>12 and interleukin 18. <i>Cytokine</i>. 1999 Nov;11(11):822-30.</p> <ul style="list-style-type: none"><li>• Abstract Inhibition of in vitro immunoglobulin production by IL-12 in murine chronic graft-vs.-host disease: synergism with IL-18. <i>Eur J Immunol</i>. 1998 Jun;28(6):2017-24.</li></ul>
IL-18/IL-21	<ul style="list-style-type: none"><li>• IL-21 in Synergy with IL-15 or IL-18 Enhances IFN-gamma Production in Human NK and T Cells. <i>J Immunol</i>. 2003 Jun 1;170(11):5464-9.</li></ul>
IL-18/TGF- $\beta$	<ul style="list-style-type: none"><li>• Interleukin 18 and transforming growth factor beta1 in the serum of patients with Graves' ophthalmopathy treated with corticosteroids. <i>Int Immunopharmacol</i>. 2003 Apr;3(4):549-52.</li></ul>
IL-18/IFN- $\gamma$	
Anti-TNF ALPHA/anti-CD4	<ul style="list-style-type: none"><li>• Synergistic therapeutic effect in DBA/1 arthritic mice.</li></ul>

**Annex 3: Oncology combinations**

Target	Disease	Pair with
CD89*	Use as cytotoxic cell recruiter	all
CD19	B cell lymphomas	HLA-DR CD5
HLA-DR	B cell lymphomas	CD89 CD19 CD5
CD38	Multiple myeloma	CD138 CD56 HLA-DR
CD138	Multiple myeloma	CD38 CD56 HLA-DR
CD138	Lung cancer	CD56 CEA
CD33	Acute myelod lymphoma	CD34 HLA-DR
CD56	Lung cancer	CD138 CEA
CEA	Pan carcinoma	MET receptor
VEGF	Pan carcinoma	MET receptor
VEGF receptor	Pan carcinoma	MET receptor
IL-13	Asthma/pulmonary inflammation	IL-4 IL-5 Eotaxin(s) MDC TARC TNF $\alpha$ IL-9 EGFR CD40L IL-25 MCP-1 TGF $\beta$
IL-4	Asthma	IL-13 IL-5 Eotaxin(s) MDC TARC TNF $\alpha$ IL-9 EGFR CD40L IL-25 MCP-1 TGF $\beta$
Eotaxin	Asthma	IL-5 Eotaxin-2 Eotaxin-3
EGFR	cancer	HER2/neu HER3 HER4
HER2	cancer	HER3

		HER4
TNFR1	RA/Crohn's disease	IL-1R IL-6R IL-18R
TNF $\alpha$	RA/Crohn's disease	IL-1 $\alpha/\beta$ IL-6 IL-18 ICAM-1 IL-15 IL-17
IL-1R	RA/Crohn's disease	IL-6R IL-18R
IL-18R	RA/Crohn's disease	IL-6R

## Annex 4

## Data Summary

[illegible]

	TAR1-5 heterodimers			With (Gly <sub>4</sub> Set) <sub>n</sub> linker TAR1-5d1 = 30nM TAR1-5d2 = 50nM TAR1-5d3 = 300nM TAR1-5d4 = 3nM TAR1-5d5 = 200nM TAR1-5d6 = 100nM  In Fab format TAR1-5CH d2CK = 30nM TAR1-5CK d3CH = 100nM  = 60nM	
	TAR1-5-19 homotrimer			0.3nM	3-10nM (eg. 3nM)
TAR2	TAR2 monomers	As TAR1 monomer		500nM to 100pM	500nM to 50pM
	TAR2-10				
	TAR2-5				
Serum Albumin	Anti-SA monomers	1nM to 500μM, preferably 100nM to 10 μM  In Dual Specific format, target affinity is 1 to 100,000 x affinity of SA dAb affinity, eg 100pM (target) and 10 μM SA affinity.		1nM to 500μM, preferably 100nM to 10 μM  In Dual Specific format, target affinity is 1 to 100,000 x affinity of SA dAb affinity, eg 100pM (target) and 10 μM SA affinity.	
	MSA-16	200nM			
	MSA-26	70nM			

Claims

1. A dual-specific ligand comprising a first immunoglobulin single variable domain having a binding specificity to a first epitope or antigen and a second complementary immunoglobulin single variable domain having a binding activity to a second epitope or antigen, wherein one or both of said antigens or epitopes acts to increase the half-life of the ligand *in vivo* and wherein said first and second domains lack mutually complementary domains which share the same specificity, provided that said dual specific ligand does not consist of an anti-HSA V<sub>H</sub> domain and an anti- $\beta$  galactosidase V<sub>K</sub> domain.
2. A dual-specific ligand according to claim 1, comprising at least one single heavy chain variable domain of an antibody and one complementary single light chain variable domain of an antibody such that the two regions are capable of associating to form a complementary V<sub>H</sub>/V<sub>L</sub> pair.
3. A dual specific ligand according to claim 2 wherein the V<sub>H</sub> and V<sub>L</sub> are provided by an antibody scFv fragment.
4. A dual-specific ligand according to claim 2 wherein the V<sub>H</sub> and V<sub>L</sub> are provided by an antibody Fab region.
5. A four chain IgG immunoglobulin ligand comprising a dual specific ligand of claim 2.
6. A four chain IgG immunoglobulin ligand according to claim 5, wherein said IgG comprises two dual specific ligands, said dual specific ligands being identical in their variable domains.
7. A four chain IgG immunoglobulin ligand according to claim 5, wherein said IgG comprises two dual specific ligands, said dual specific ligands being different in their variable domains.
8. A ligand comprising a first immunoglobulin variable domain having a first antigen or epitope binding specificity and a second immunoglobulin variable domain having a second antigen or epitope binding specificity wherein one or both of said first and second variable domains bind to an antigen which increases the half-life of the ligand *in vivo*, and the variable domains are not complementary to one another.
9. A ligand according to claim 8 wherein the first and the second immunoglobulin variable domains are heavy chain variable domains (V<sub>H</sub>).
10. A ligand according to claim 8 wherein the first and the second immunoglobulin variable domains are light chain variable domains (V<sub>L</sub>).
11. A ligand according to claim any preceding claim, wherein the first and second epitopes bind independently, such that the dual specific ligand may simultaneously bind both the first and second epitopes or antigens.

12. A ligand according to claim 11, wherein the dual specific ligand comprises a first form and a second form in equilibrium in solution, wherein both epitopes or antigens bind to the first form independently but compete for binding to the second form.
13. A ligand according to any preceding claim wherein the variable regions are derived from immunoglobulins directed against said epitopes or antigens.
14. A ligand according to any preceding claim, wherein said first and second epitopes are present on separate antigens.
15. A ligand according to any one of claims 1 to 11, wherein said first and second epitopes are present on the same antigen.
16. A ligand according to any preceding claim comprising a variable domain that is derived from a repertoire of single antibody domains.
17. A ligand of claim 16 wherein said repertoire is displayed on the surface of filamentous bacteriophage and wherein the single antibody domains are selected by binding of the bacteriophage repertoire to antigen.
18. A ligand according to any preceding claim wherein the sequence of at least one variable domain is modified by mutation or DNA shuffling.
19. A dual-specific ligand according to any preceding claim wherein the variable regions are non-covalently associated.
20. A dual-specific ligand according to any one of claims 1 to 18 wherein the variable regions are covalently associated.
21. A dual-specific ligand according to claim 20 wherein the covalent association is mediated by disulphide bonds.
22. A dAb monomer ligand specific for  $\text{TNF}\alpha$ , which dissociates from human  $\text{TNF}\alpha$  with a dissociation constant ( $K_d$ ) of 50nM to 20pM, and a  $K_{\text{off}}$  rate constant of  $5 \times 10^{-1}$  to  $1 \times 10^{-7} \text{ s}^{-1}$ , as determined by surface plasmon resonance.
23. A dAb monomer ligand specific for  $\text{TNF}\alpha$  according to claim 22, wherein the dAb is a  $V_{\kappa}$ .
24. A dAb monomer ligand specific for TNF receptor 1 (p55), which dissociates from human TNF receptor 1 with a dissociation constant ( $K_d$ ) of 50nM to 20pM, and a  $K_{\text{off}}$  rate constant of  $5 \times 10^{-1}$  to  $1 \times 10^{-7} \text{ s}^{-1}$ , as determined by surface plasmon resonance.
25. A dAb monomer ligand according to claim 22 to claim 24, wherein the monomer neutralises human  $\text{TNF}\alpha$  or TNF receptor 1 in a standard cell assay with an ND50 of 500nM to 50pM.



26. A dAb monomer ligand specific for TNF receptor 1 (p55), wherein the dAb antagonises the activity of the TNF receptor 1 in a standard cell assay with an  $ND_{50}$  of  $\leq 100\text{nM}$ , and at a concentration of  $\leq 10\mu\text{M}$  the dAb agonises the activity of the TNF receptor 1 by  $\leq 5\%$  in the assay.
27. A dAb monomer ligand specific for serum albumin (SA) which dissociates from SA with a dissociation constant ( $K_d$ ) of  $1\text{nM}$  to  $500\mu\text{M}$ , as determined by surface plasmon resonance.
28. A dAb monomer ligand according to claim 27, wherein the monomer binds SA in a standard ligand binding assay with an  $IC_{50}$  of  $1\text{nM}$  to  $500\mu\text{M}$ .
29. A dAb monomer ligand specific for  $\text{TNF}\alpha$ , wherein the dAb comprises the amino acid sequence of TAR1-5-19 or a sequence that is at least 80% homologous thereto.
30. A dAb monomer ligand specific for  $\text{TNF}\alpha$ , wherein the dAb comprises the amino acid sequence of TAR1-5 or a sequence that is at least 80% homologous thereto.
31. A dAb monomer ligand specific for  $\text{TNF}\alpha$ , wherein the dAb comprises the amino acid sequence of TAR1-27 or a sequence that is at least 80% homologous thereto.
32. A dAb monomer ligand specific for TNF receptor 1, wherein the dAb comprises the amino acid sequence of TAR2-10 or a sequence that is at least 80% homologous thereto.
33. A dAb monomer ligand specific for TNF receptor 1, wherein the dAb comprises the amino acid sequence of TAR2-10 or a sequence that is at least 90% homologous thereto.
34. A dAb monomer ligand specific for TNF receptor 1, wherein the dAb comprises the amino acid sequence of TAR2-5 or a sequence that is at least 80% homologous thereto.
35. A dAb monomer ligand specific for TNF receptor 1, wherein the dAb comprises the amino acid sequence of TAR2-5 or a sequence that is at least 90% homologous thereto.
36. A dAb monomer ligand specific for SA, wherein the dAb comprises the amino acid sequence of MSA-16 or a sequence that is at least 80% homologous thereto.
37. A dAb monomer ligand specific for SA, wherein the dAb comprises the amino acid sequence of MSA-26 or a sequence that is at least 80% homologous thereto.

38. A dAb monomer according to any one of claims 29 to 37, wherein the TNF $\alpha$ , TNF receptor 1 or SA is in human form.
39. A dAb monomer further comprising a terminal Cys residue.
40. A dAb monomer according to any one of claims 29 to 38, further comprising a terminal Cys residue.
41. A dual specific ligand comprising at least one dAb monomer according to any one of claims 22 to 40.
42. A dual specific ligand according to claim 41, which is a dimer.
43. A dual specific ligand according to claim 42, wherein the dimer comprises anti-human TNF alpha dAb according to any one of claims 22, 23 and 28-30, and an anti-SA dAb according to any one of claims 26, 27 and 34-36.
44. A dual specific ligand according to claim 42, wherein the dimer is a homo- or hetero-dimer comprising first and second anti-human TNF alpha dAbs, each dAb being according to any one of claims 22, 23 and 28-30.
45. A dual specific ligand according to claim 41, which is a trimer.
46. A dual specific ligand according to claim 45, which is a homotrimer comprising three copies of an anti-human TNF alpha dAb according to any one of claims 22, 23 and 29-31.
47. A ligand according to any preceding claim, which comprises a universal framework.
48. A ligand according to claim 47, wherein the universal framework comprises a V<sub>H</sub> framework selected from the group consisting of DP47, DP45 and DP38; and/or the V<sub>L</sub> framework is DPK9.
49. A ligand according to any preceding claim which comprises a binding site for a generic ligand.
50. The ligand of claim 49, wherein the generic ligand binding site is selected from the group consisting of protein A, protein L and protein G.
51. A ligand according to any preceding claim, wherein the ligand comprises a variable domain having one or more framework regions comprising an amino acid sequence that is the same as the amino acid sequence of a corresponding framework region encoded by a human germline antibody gene segment, or the amino acid sequences of one or more of said framework regions collectively comprises up to 5 amino acid differences relative to the amino acid sequence of said corresponding framework region encoded by a human germline antibody gene segment.

52. A ligand according to any one of claims 1 to 51, wherein the ligand comprises a variable domain, wherein the amino acid sequences of FW1, FW2, FW3 and FW4 are the same as the amino acid sequences of corresponding framework regions encoded by a human germline antibody gene segment, or the amino acid sequences of FW1, FW2, FW3 and FW4 collectively contain up to 10 amino acid differences relative to the amino acid sequences of corresponding framework regions encoded by said human germline antibody gene segment.

53. The ligand according to claim 51 or claim 52, which comprises an antibody variable domain comprising FW1, FW2 and FW3 regions, and the amino acid sequence of said FW1, FW2 and FW3 are the same as the amino acid sequences of corresponding framework regions encoded by human germline antibody gene segments.

54. The ligand according to any one of claims 51 to 53, wherein said human germline antibody gene segment is selected from the group consisting of DP47, DP45, DP48 and DPK9.

55. A ligand according to any preceding claim, comprising a V<sub>H</sub> domain that is not a Camelid immunoglobulin variable domain.

56. The ligand of Claim 55, comprising a V<sub>H</sub> domain that does not contain one or more amino acids that are specific to Camelid immunoglobulin variable domains as compared to human V<sub>H</sub> domains.

57. A method for producing a ligand comprising a first immunoglobulin single variable domain having a first binding specificity and a second single immunoglobulin single variable domain having a second binding specificity, one or both of the binding specificities being specific for a protein which increases the half-life of the ligand *in vivo*, the method comprising the steps of:

- (a) selecting a first variable domain by its ability to bind to a first epitope,
- (b) selecting a second variable region by its ability to bind to a second epitope,
- (c) combining the variable regions; and
- (d) selecting the ligand by its ability to bind to said first and second epitopes; wherein, when said variable domains are complementary, neither of said domains is a V<sub>H</sub> domain specific for HSA.

58. A method according to claim 57 wherein said first variable domain is selected for binding to said first epitope in absence of a complementary variable domain.

59. A method according to claim 57 wherein said first variable domain is selected for binding to said first epitope in the presence of a third complementary variable domain in which said third variable domain is different from said second variable domain.

60. Nucleic acid encoding a dual-specific ligand according to any one of claims 1 to 56.

61. A nucleic acid according to claim 60 which is specific for TNF $\alpha$ , comprising the nucleic acid sequence of TAR1-5-19 or a sequence that is at least 70% homologous thereto.
62. A nucleic acid according to claim 60 which is specific for TNF $\alpha$ , comprising the nucleic acid sequence of TAR1-5 or a sequence that is at least 70% homologous thereto.
63. A nucleic acid according to claim 60 which is specific for TNF $\alpha$ , comprising the nucleic acid sequence of TAR1-27 or a sequence that is at least 70% homologous thereto.
64. A nucleic acid according to claim 60 which is specific for TNF receptor 1, comprising the nucleic acid sequence of TAR2-10 or a sequence that is at least 70% homologous thereto.
65. A nucleic acid according to claim 60 which is specific for TNF receptor 1, comprising the nucleic acid sequence of TAR2-10 or a sequence that is at least 80% homologous thereto.
66. A nucleic acid according to claim 60 which is specific for TNF receptor 1, comprising the nucleic acid sequence of TAR2h-5 or a sequence that is at least 70% homologous thereto.
67. A nucleic acid according to claim 60 which is specific for TNF receptor 1, comprising the nucleic acid sequence of TAR2h-5 or a sequence that is at least 80% homologous thereto.
68. A nucleic acid according to claim 60 which is specific for SA, comprising the nucleic acid sequence of MSA-16 or a sequence that is at least 70% homologous thereto.
69. A nucleic acid according to claim 60 which is specific for SA, comprising the nucleic acid sequence of MSA-26 or a sequence that is at least 70% homologous thereto.
70. A vector comprising nucleic acid according to any one of claims 60 to 69.
71. A vector according to claim 70, further comprising components necessary for the expression of a dual-specific ligand.
72. A host cell transfected with a vector according to claim 71.
73. A method for producing a closed conformation multi-specific ligand comprising a first single epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity, wherein the first and second binding specificities are capable of competing for epitope binding such that the closed

conformation multi-specific ligand may not bind both epitopes simultaneously, said method comprising the steps of:

- a) selecting a first epitope binding domain by its ability to bind to a first epitope,
- b) selecting a second epitope binding domain by its ability to bind to a second epitope,
- c) combining the epitope binding domains such that the domains are in a closed conformation; and
- d) selecting the closed conformation multispecific ligand by its ability to bind to said first second epitope and said second epitope, but not to both said first and second epitopes simultaneously.

74. A method according to claim 73 wherein the first and the second epitope binding domains are immunoglobulin variable heavy chain domains ( $V_H$ ).

75. A method according to claim 73 wherein the first and the second immunoglobulin variable domains are immunoglobulin variable light chain domains ( $V_L$ ).

76. A method according to any one of claims 73 to 75 wherein the immunoglobulin domains are derived from immunoglobulins directed against said epitopes.

77. A method according to any one of claims 73 to 76, wherein said first and second epitopes are present on separate antigens.

78. A method according to any one of claims 73 to 76, wherein said first and second epitopes are present on the same antigen.

79. A method according to any one of claims 73 to 78 wherein the variable domain is derived from a repertoire of single antibody domains.

80. A method of claim 79 wherein said repertoire is displayed on the surface of filamentous bacteriophage and wherein the single antibody domains are selected by binding of the bacteriophage repertoire to antigen.

81. A method of any one of claims 73 to 80 wherein the sequence of at least one immunoglobulin variable domain is modified by mutation or DNA shuffling.

82. A closed conformation multispecific ligand comprising a first epitope binding domain having a first epitope binding specificity and a non-complementary second epitope binding domain having a second epitope binding specificity wherein the first and second binding specificities are capable of competing for epitope binding such that the closed conformation multi-specific ligand cannot bind both epitopes simultaneously.

83. A closed conformation multispecific ligand according to claim 82, obtainable by a method according to any one of claims 73 to 80.

84. A closed conformation multispecific ligand according to claim 82 or claim 83, comprising more than one single heavy chain variable domain of an antibody or more than one light chain variable domain of an antibody.
85. A closed conformation multi-specific ligand according to claim 84 wherein the  $V_H$  and  $V_L$  are linked by a peptide linker.
86. A closed conformation multi-specific ligand according to claim 84 wherein the  $V_H$  or  $V_L$  are provided by an antibody Fab-like region.
87. A closed conformation multi-specific ligand according to any one of claims 82 to 84 wherein the variable regions are non-covalently associated.
88. A closed conformation multi-specific ligand according to any one of claims 82 to 84 wherein the variable regions are covalently associated.
89. A closed conformation multi-specific ligand according to claim 87 wherein the covalent association is mediated by disulphide bonds.
90. A closed conformation multi-specific ligand according to any of claims 82 to 89 which comprises a universal framework.
91. A closed conformation multi-specific ligand according to any of claims 82 to 90 which comprises a binding site for a generic ligand.
92. The closed conformation multi-specific ligand of claim 91, wherein the generic ligand binding site is selected from the group consisting of protein A, protein L and protein G.
93. A closed conformation ligand according to any of claims 82 to 92, wherein the ligand comprises a variable domain having one or more framework regions comprising an amino acid sequence that is the same as the amino acid sequence of a corresponding framework region encoded by a human germline antibody gene segment, or the amino acid sequences of one or more of said framework regions collectively comprises up to 5 amino acid differences relative to the amino acid sequence of said corresponding framework region encoded by a human germline antibody gene segment.
94. The closed conformation ligand according to claim 93, wherein the ligand comprises a variable domain wherein the amino acid sequences of FW1, FW2, FW3 and FW4 are the same as the amino acid sequences of corresponding framework regions encoded by a human germline antibody gene segment, or the amino acid sequences of FW1, FW2, FW3 and FW4 collectively contain up to 10 amino acid differences relative to the amino acid sequences of corresponding framework regions encoded by said human germline antibody gene segment.
95. The closed conformation ligand according to claim 93 or claim 94, which comprises an antibody variable domain comprising FW1, FW2 and FW3 regions,

and the amino acid sequence of said FW1, FW2 and FW3 are the same as the amino acid sequences of corresponding framework regions encoded by human germline antibody gene segments.

96. The closed conformation ligand according to any one of claims 92 to 95, wherein said human germline antibody gene segment is selected from the group consisting of DP47, DP45, DP48 and DPK9.

97. A closed conformation ligand according to any one of claims 92 to 96, comprising a V<sub>H</sub> domain that is not a Camelid immunoglobulin variable domain.

98. The closed conformation ligand of Claim 97, wherein the V<sub>H</sub> domain does not contain one or more amino acids that are specific to Camelid immunoglobulin variable domains as compared to human V<sub>H</sub> domains.

99. A closed conformation multi-specific ligand according to any one of claims 82 to 98, wherein one specificity thereof is for an agent effective to increase the half life of the ligand.

100. A kit comprising a closed conformation multi-specific ligand according to any one of claims 82 to 99.

101. Nucleic acid encoding at least a closed conformation multispecific ligand according to any one of claims 82 to 99.

102. A vector comprising nucleic acid according to claim 101.

103. A vector according to claim 102, further comprising components necessary for the expression of a closed conformation multispecific ligand.

104. A host cell transfected with a vector according to claim 103.

105. A method for detecting the presence of a target molecule, comprising:  
(a) providing a closed conformation multispecific ligand bound to an agent, said ligand being specific for the target molecule and the agent, wherein the agent which is bound by the ligand leads to the generation of a detectable signal on displacement from the ligand;  
(b) exposing the closed conformation multispecific ligand to the target molecule; and  
(c) detecting the signal generated as a result of the displacement of the agent.

106. A method according to claim 105, wherein the agent is an enzyme, which is inactive when bound by the closed conformation multispecific ligand.

107. A method according to claim 105, wherein the agent is the substrate for an enzyme

108. A method according to claim 107, wherein the agent is a fluorescent, luminescent or chromogenic molecule which is inactive or quenched when bound by the ligand.

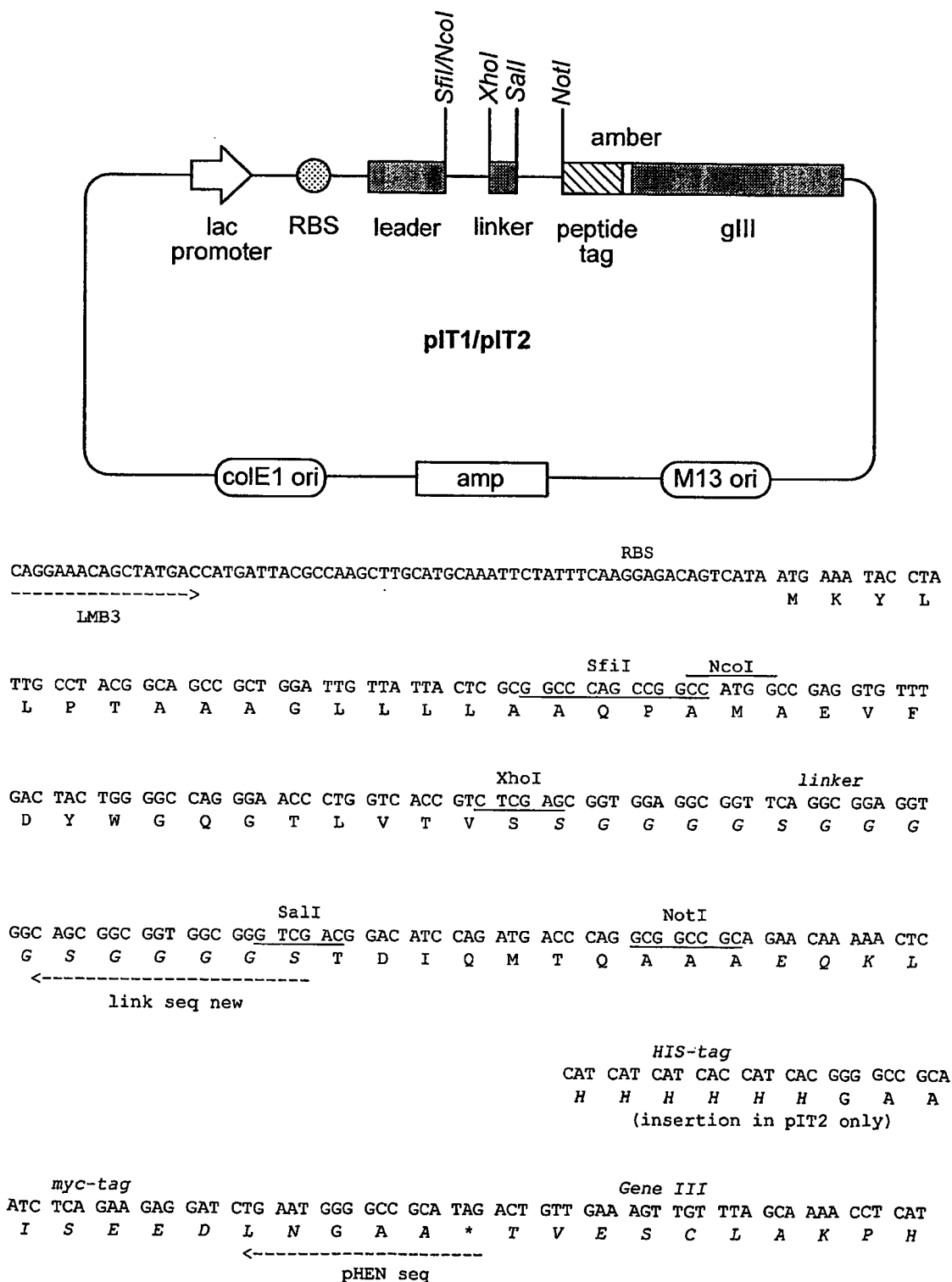
109. A kit for performing a method according to any one of claims 105-108, comprising a closed conformation multispecific ligand capable of binding to a target molecule, and optionally an agent and buffers suitable therefor.
110. A homogenous immunoassay incorporating a method according to any one of claims 105-108.
111. A ligand according to any one of claims 1 to 56 for use in therapy.
112. A pharmaceutical composition comprising a ligand according to any one of claims 1 to 56, and a pharmaceutically acceptable excipient, carrier or diluent.
113. A method for preparing a chelating multimeric ligand comprising the steps of:
- (a) providing a vector comprising a nucleic acid sequence encoding a single binding domain specific for a first epitope on a target;
  - (b) providing a vector encoding a repertoire comprising second binding domains specific for a second epitope on said target, which epitope can be the same or different to the first epitope, said second epitope being adjacent to said first epitope; and
  - (c) expressing said first and second binding domains; and
  - (d) isolating those combinations of first and second binding domains which combine together to produce a target-binding dimer.
114. A method according to claim 113, wherein the first and second binding domains are associated covalently through a linker.
115. A method according to claim 113, wherein the first and second binding domains are associated non-covalently.
116. A method according to claim 113, wherein the first and second binding domains are associated through natural association of the domains.
117. A method according to claim 116, wherein the binding domains comprise a  $V_H$  domain and a  $V_K$  domain.





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FIG. 2



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FIG. 3

V <sub>H</sub> chains												
	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4					
	1	2	3	4	5	6	7	8	9	10	11	
	123456789012345678901234567890	12345	67890123456789	012a3456789012345	67890123456789012abc345678901234	567801	234567890123					
V <sub>H</sub> dummy	EVQLLESGGGLVQPGGSLRLSRAAGFTFS	SYAMS	WVRQAPGKLEWVS	AISGSGSTYYADSVKG	RFTISRDNKNTLYLQMNSRAEDTAVYYCAK	SYGAFDY	WGQGTLLVTVSS					
K8	-----	-----	-----	H-SPY-AN-R-----	-----	GIRA-----	-----					
VH2	-----	-----	-----	D-GAT-SK-G---P---	-----	KVLT-----	-----					
VH4	-----	-----	-----	R-NGP--*A-G-----	-----I-----	HGAP-----	-----					
VHC11	-----	---N-----	-----	S-PAS-LH-R-----	-----	PGLG-----	-----					
VHA10sd	-----	-----	-----	D-ERT-Y*-R-----	-----	KVLV-----	-----					
VHA1sd	-----	-----	-----	E-SAN-SK-Q-----	-L-----	KVLQ-----	-----					
VHA5sd	-----	-----	-----	T-PAN-*V-R-----	-----	SLLQ-----	-----					
VHC5sd	-----	-----	-----	D-AAT-SA-S-----	-----	KILK-----	-----					
VHC11sd	-----S-----	-----	-----	T-SSV-QS-R-----	-----	NLMS-----	-----					
V <sub>K</sub> chains												
	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4					
	1	2	3	4	5	6	7	8	9	10		
	12345678901234567890123	45678901234	567890123456789	0123456789012345678	78901234567890123456789012345678	901234567	89012345678					
V <sub>K</sub> dummy	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTPNT	FGQGTKEIKR					
K8	-----	-----	-----	R-H-----	-----	--PQRS-G-	-----					
E5sd	-----	---V-----	-----	L-R-----	-----	--NWL-P-	-----					
C3	-----	-----	-----	*-L-----	-----	--RYVD-L-	-----					

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FIG. 4

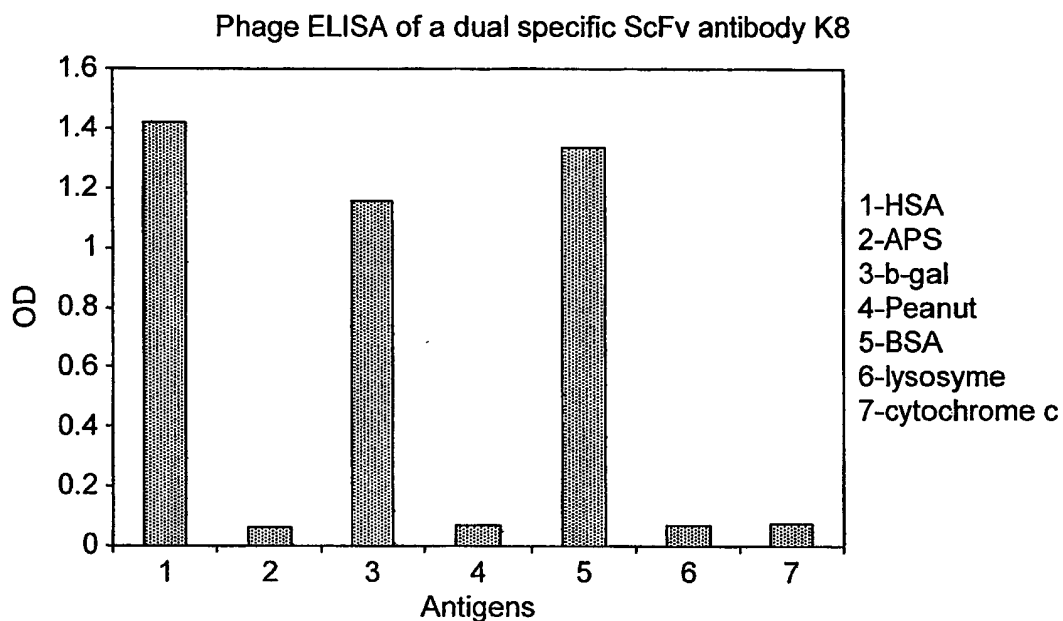
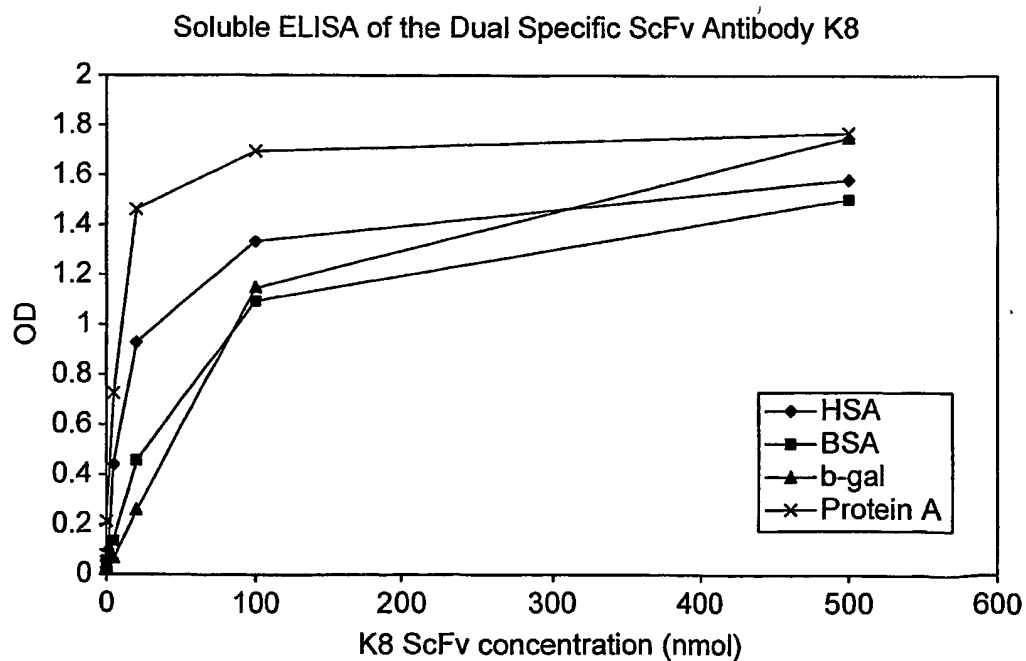
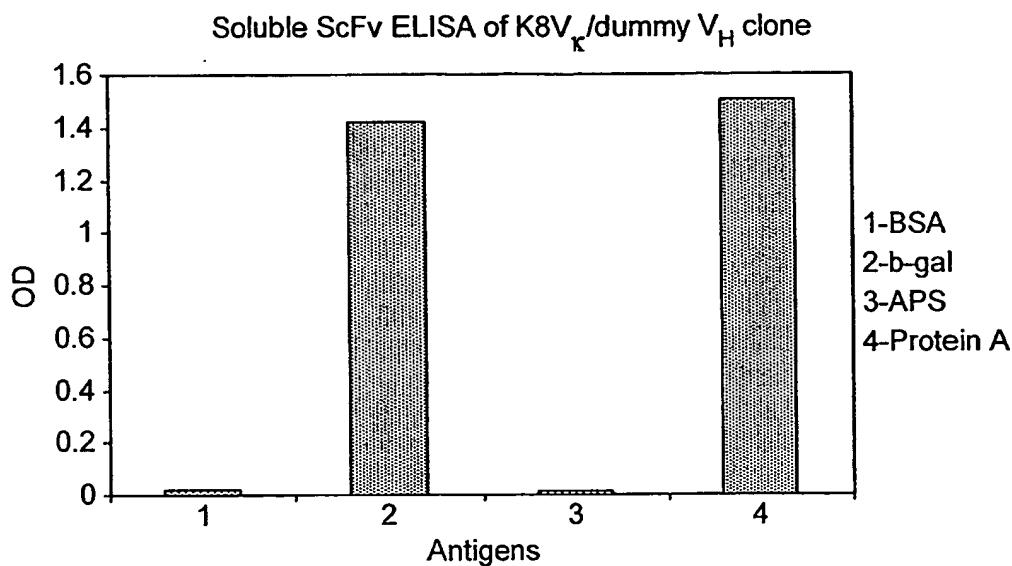


FIG. 5



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## FIG. 6



## FIG. 7

RBS

CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATTCATTTCAAGGAGACAGTCATA ATG AAA TAC CTA  
 -----> M K Y L

LMB3

SfiI                      NcoI

TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC GAG GTG TTT  
 L P T A A A G L L L L A A Q P A M A E V F

XhoI                      linker

GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GGT GGA GGC GGT TCA GGC GGA GGT  
 D Y W G Q G T L V T V S S G G G G S G G G

SalI                      NotI

GGC AGC GGC GGT GGC GGG TCG ACG GAC ATC CAG ATG ACC CAG GCG GCC GCA GAA CAA AAA CTC ..  
 G S G G G G S T D I Q M T Q A A A E Q K L

<-----  
 link seq new

HIS-tag

CAT CAT CAT CAC CAT CAC GGG GCC GCA  
 H H H H H H G A A  
 (insertion in V domain vector 2 only)

myc-tag                      Gene III

ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG ACT GTT GAA AGT TGT TTA GCA AAA CCT CAT  
 I S E E D L N G A A \* T V E S C L A K P H

<-----  
 pHEN seq

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FIG. 8

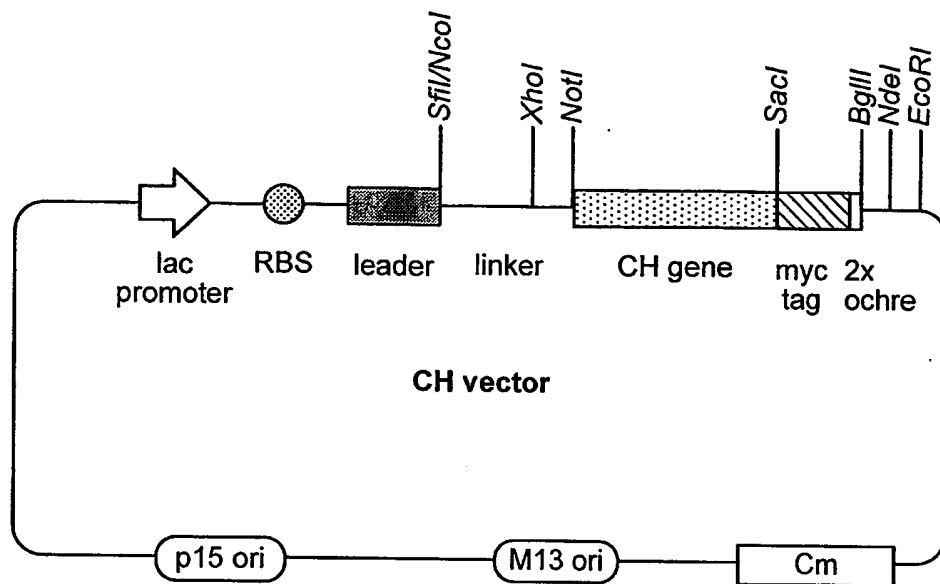
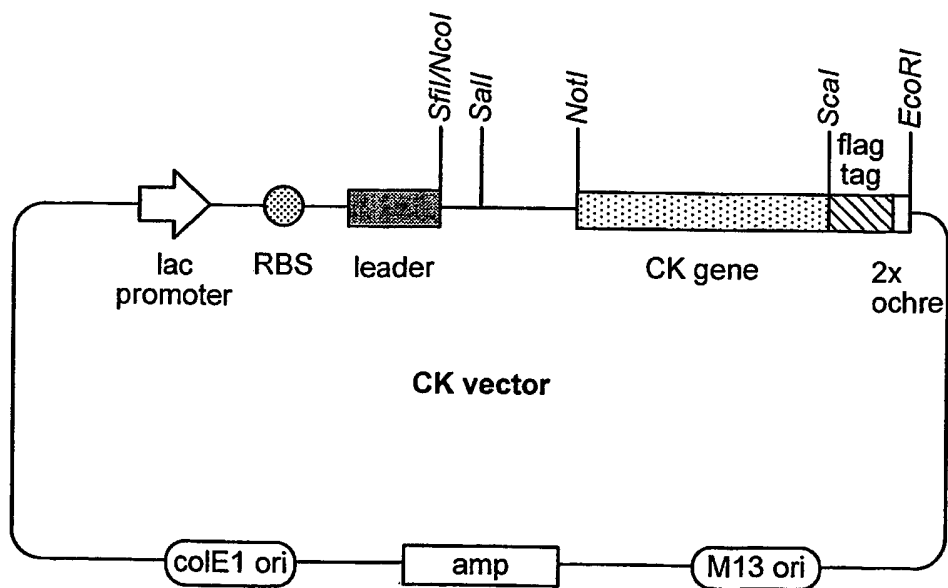


FIG. 9



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FIG. 10

TNF receptor assay

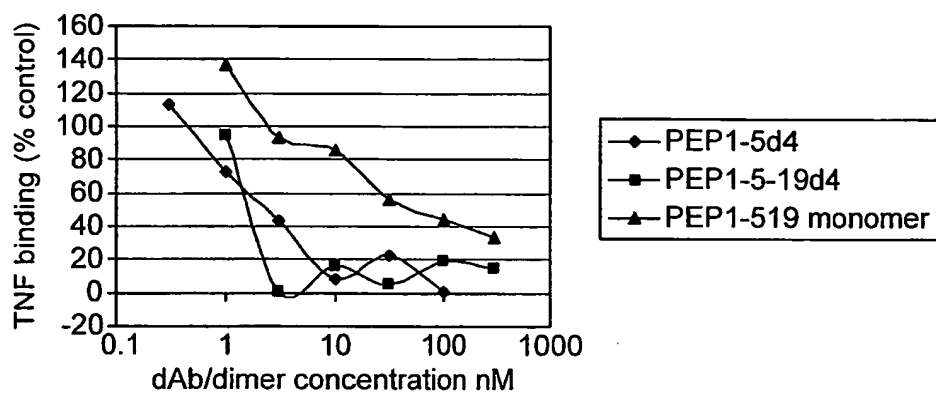


FIG. 11

TNF receptor assay

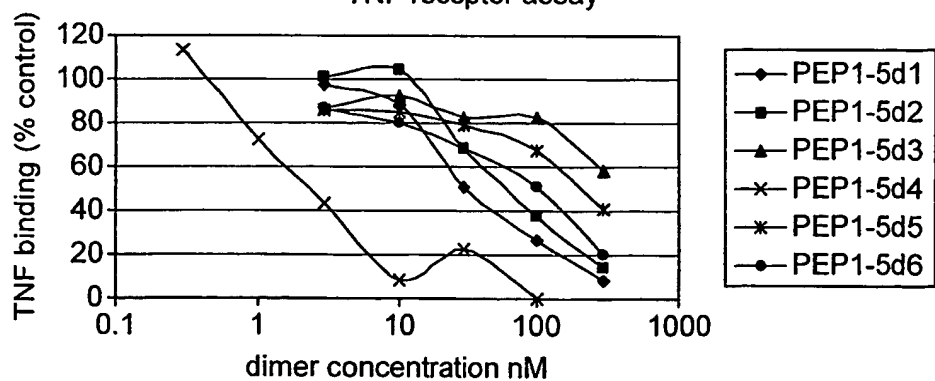
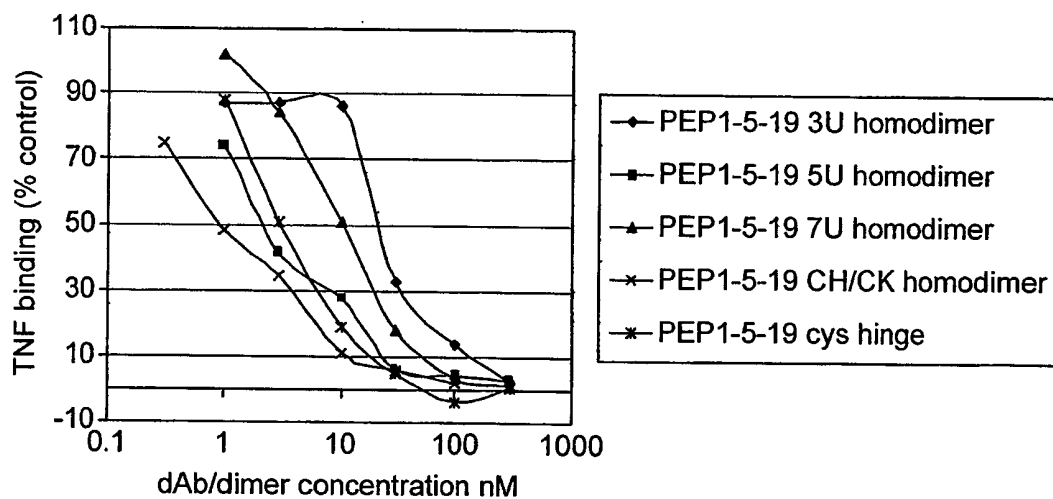


FIG. 12

TNF Receptor assay





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## FIG. 13

Dummy V<sub>H</sub> sequence for library 1

1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G
	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGG
	CTC	CAC	GTC	GAC	AAC	CTC	AGA	CCC	CCT	CCG	AAC	CAT	GTC	GGA	CCC	CCC
49	S	L	R	L	S	C	A	A	S	G	F	T	F	<u>S</u>	<u>S</u>	Y
	TCC	CTG	CGT	CTC	TCC	TGT	GCA	GCC	TCC	GGA	TTC	ACC	TTT	<u>AGC</u>	<u>AGC</u>	TAT
	AGG	GAC	GCA	GAG	AGG	ACA	CGT	CGG	AGG	CCT	AAG	TGG	AAA	<u>TCG</u>	<u>TCG</u>	ATA
97	<u>A</u>	M	<u>S</u>	W	V	R	Q	A	P	G	K	G	L	E	W	V
	<u>GCC</u>	ATG	<u>AGC</u>	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGT	CTA	GAG	TGG	GTC
	<u>CGG</u>	TAC	<u>TCG</u>	ACC	CAG	GCG	GTC	CGA	GGT	CCC	TTC	CCA	GAT	CTC	ACC	CAG
145	S	<u>A</u>	I	<u>S</u>	<u>G</u>	<u>S</u>	G	<u>G</u>	<u>S</u>	T	<u>Y</u>	Y	A	D	S	V
	TCA	<u>GCT</u>	ATT	<u>AGT</u>	<u>GGT</u>	<u>AGT</u>	GGT	<u>GGT</u>	<u>AGC</u>	ACA	<u>TAC</u>	TAC	GCA	GAC	TCC	GTG
	AGT	<u>CGA</u>	TAA	<u>TCA</u>	<u>CCA</u>	<u>TCA</u>	CCA	<u>CCA</u>	<u>TCG</u>	TGT	<u>ATG</u>	ATG	CGT	CTG	AGG	CAC
193	K	G	R	F	T	I	S	R	D	N	S	K	N	T	L	Y
	AAG	GGC	CGG	TTC	ACC	ATC	TCC	CGT	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT
	TTC	CCG	GCC	AAG	TGG	TAG	AGG	GCA	CTG	TTA	AGG	TTC	TTG	TGC	GAC	ATA
241	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C
	CTG	CAA	ATG	AAC	AGC	CTG	CGT	GCC	GAG	GAC	ACC	GCG	GTA	TAT	TAC	TGT
	GAC	GTT	TAC	TTG	TCG	GAC	GCA	CGG	CTC	CTG	TGG	CGC	CAT	ATA	ATG	ACA
289	A	K	<u>S</u>	<u>Y</u>	<u>G</u>	<u>A</u>	F	D	Y	W	G	Q	G	T	L	V
	GCG	AAA	<u>AGT</u>	<u>TAT</u>	<u>GGT</u>	<u>GCT</u>	TTT	GAC	TAC	TGG	GGC	CAG	GGA	ACC	CTG	GTC
	CGC	TTT	<u>TCA</u>	<u>ATA</u>	<u>CCA</u>	<u>CGA</u>	AAA	CTG	ATG	ACC	CCG	GTC	CCT	TGG	GAC	CAG
337	T	V	S	S												
	ACC	GTC	TCG	AGC												
	TGG	CAG	AGC	TCG												

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## FIG. 14

Dummy V<sub>H</sub> sequence for library 2

1	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G	G
	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGG
	CTC	CAC	GTC	GAC	AAC	CTC	AGA	CCC	CCT	CCG	AAC	CAT	GTC	GGA	CCC	CCC
49	S	L	R	L	S	C	A	A	S	G	F	T	F	S	S	Y
	TCC	CTG	CGT	CTC	TCC	TGT	GCA	GCC	TCC	GGA	TTC	ACC	TTT	<u>AGC</u>	<u>AGC</u>	TAT
	AGG	GAC	GCA	GAG	AGG	ACA	CGT	CGG	AGG	CCT	AAG	TGG	AAA	<u>TCG</u>	<u>TCG</u>	ATA
97	<u>A</u>	M	<u>S</u>	W	V	R	Q	A	P	G	K	G	L	E	W	V
	<u>GCC</u>	ATG	<u>AGC</u>	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGT	CTA	GAG	TGG	GTC
	<u>CGG</u>	TAC	<u>TCG</u>	ACC	CAG	GCG	GTC	CGA	GGT	CCC	TTC	CCA	GAT	CTC	ACC	CAG
145	S	<u>A</u>	I	<u>S</u>	<u>G</u>	<u>S</u>	G	<u>G</u>	<u>S</u>	T	<u>Y</u>	Y	A	D	S	V
	TCA	<u>GCT</u>	ATT	<u>AGT</u>	<u>GGT</u>	<u>AGT</u>	GGT	<u>GGT</u>	<u>AGC</u>	ACA	<u>TAC</u>	TAC	GCA	GAC	TCC	GTG
	AGT	<u>CGA</u>	TAA	<u>TCA</u>	<u>CCA</u>	<u>TCA</u>	CCA	<u>CCA</u>	<u>TCG</u>	TGT	<u>ATG</u>	ATG	CGT	CTG	AGG	CAC
193	K	G	R	F	T	I	S	R	D	N	S	K	N	T	L	Y
	AAG	GGC	CGG	TTC	ACC	ATC	TCC	CGT	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT
	TTC	CCG	GCC	AAG	TGG	TAG	AGG	GCA	CTG	TTA	AGG	TTC	TTG	TGC	GAC	ATA
241	L	Q	M	N	S	L	R	A	E	D	T	A	V	Y	Y	C
	CTG	CAA	ATG	AAC	AGC	CTG	CGT	GCC	GAG	GAC	ACC	GCG	GTA	TAT	TAC	TGT
	GAC	GTT	TAC	TTG	TCG	GAC	GCA	CGG	CTC	CTG	TGG	CGC	CAT	ATA	ATG	ACA
289	A	K	<u>S</u>	<u>Y</u>	<u>G</u>	<u>A</u>	<u>X</u>	<u>X</u>	<u>X</u>	<u>X</u>	F	D	Y	W	G	Q
	GCG	AAA	<u>AGT</u>	<u>TAT</u>	<u>GGT</u>	<u>GCT</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	TTT	GAC	TAC	TGG	GGC	CAG
	CGC	TTT	<u>TCA</u>	<u>ATA</u>	<u>CCA</u>	<u>CGA</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	<u>NNK</u>	AAA	CTG	ATG	ACC	CCG	GTC
337	G	T	L	V	T	V	S	S								
	GGA	ACC	CTG	GTC	ACC	GTC	TCG	AGC								
	CCT	TGG	GAC	CAG	TGG	CAG	AGC	TCG								

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## FIG. 15

Dummy V<sub>k</sub> sequence for library 3

1	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V	G
	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT	GTA	GGA
	CTG	TAG	GTC	TAC	TGG	GTC	AGA	GGT	AGG	AGG	GAC	AGA	CGT	AGA	CAT	CCT
49	D	R	V	T	I	T	C	R	A	S	Q	S	I	<u>S</u>	<u>S</u>	<u>Y</u>
	GAC	CGT	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG	AGC	ATT	<u>AGC</u>	<u>AGC</u>	<u>TAT</u>
	CTG	GCA	CAG	TGG	TAG	TGA	ACG	GCC	CGT	TCA	GTC	TCG	TAA	<u>TCG</u>	<u>TCG</u>	<u>ATA</u>
97	L	<u>N</u>	W	Y	Q	Q	K	P	G	K	A	P	K	L	L	I
	TTA	<u>AAT</u>	TGG	TAC	CAG	CAG	AAA	CCA	GGG	AAA	GCC	CCT	AAG	CTC	CTG	ATC
	AAT	<u>TTA</u>	ACC	ATG	GTC	GTC	TTT	GGT	CCC	TTT	CGG	GGA	TTC	GAG	GAC	TAG
145	Y	<u>A</u>	A	S	<u>S</u>	L	Q	S	G	V	P	S	R	F	S	G
	TAT	<u>GCT</u>	GCA	TCC	<u>AGT</u>	TTG	CAA	AGT	GGG	GTC	CCA	TCA	CGT	TTC	AGT	GGC
	ATA	<u>CGA</u>	CGT	AGG	<u>TCA</u>	AAC	GTT	TCA	CCC	CAG	GGT	AGT	GCA	AAG	TCA	CCG
193	S	G	S	G	T	D	F	T	L	T	I	S	S	L	Q	P
	AGT	GGA	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	CAA	CCT
	TCA	CCT	AGA	CCC	TGT	CTA	AAG	TGA	GAG	TGG	TAG	TCG	TCA	GAC	GTT	GGA
241	E	D	F	A	T	Y	Y	C	Q	Q	<u>S</u>	<u>Y</u>	<u>S</u>	<u>T</u>	P	<u>N</u>
	GAA	GAT	TTT	GCT	ACG	TAC	TAC	TGT	CAA	CAG	<u>AGT</u>	<u>TAC</u>	<u>AGT</u>	<u>ACC</u>	CCT	<u>AAT</u>
	CTT	CTA	AAA	CGA	TGC	ATG	ATG	ACA	GTT	GTC	<u>TCA</u>	<u>ATG</u>	<u>TCA</u>	<u>TGG</u>	GGA	<u>TTA</u>
289	T	F	G	Q	G	T	K	V	E	I	K	R				
	ACG	TTC	GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG				
	TGC	AAG	CCG	GTT	CCC	TGG	TTC	CAC	CTT	TAG	TTT	GCC				

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## FIG. 16

Nucleotide and amino acid sequence of anti MSA dAbs MSA 16 and MSA 26

## A: MSA 16

```

GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT
D   I   Q   M   T   Q   S   P   S   S   L   S   A   S
GTA GGA GAC CGT GTC ACC ATC ACT TGC CGG GCA AGT CAG AGC
V   G   D   R   V   T   I   T   C   R   A   S   Q   S
ATT ATT AAG CAT TTA AAG TGG TAC CAG CAG AAA CCA GGG AAA
I   I   K   H   L   K   W   ... Y   Q   Q   K   P   G   K
GCC CCT AAG CTC CTG ATC TAT GGT GCA TCC CGG TTG CAA AGT
A   P   K   L   L   I   Y   G   A   S   R   L   Q   S
GGG GTC CCA TCA CGT TTC AGT GGC AGT GGA TCT GGG ACA GAT
G   V   P   S   R   F   S   G   S   G   S   G   T   D
TTC ACT CTC ACC ATC AGC AGT CTG CAA CCT GAA GAT TTT GCT
F   T   L   T   I   S   S   L   Q   P   E   D   F   A
ACG TAC TAC TGT CAA CAG GGG GCT CGG TGG CCT CAG ACG TTC
T   Y   Y   C   Q   Q   G   A   R   W   P   Q   T   F
GGC CAA GGG ACC AAG GTG GAA ATC AAA CGG
G   Q   G   T   K   V   E   I   K   R

```

## B: MSA 26

```

GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT
D   I   Q   M   T   Q   S   P   S   S   L   S   A   S
GTA GGA GAC CGT GTC ACC ATC ACT TGC CGG GCA AGT CAG AGC
V   G   D   R   V   T   I   T   C   R   A   S   Q   S
ATT TAT TAT CAT TTA AAG TGG TAC CAG CAG AAA CCA GGG AAA
I   Y   Y   H   L   K   W   Y   Q   Q   K   P   G   K
GCC CCT AAG CTC CTG ATC TAT AAG GCA TCC ACG TTG CAA AGT
A   P   K   L   L   I   Y   K   A   S   T   L   Q   S
GGG GTC CCA TCA CGT TTC AGT GGC AGT GGA TCT GGG ACA GAT
G   V   P   S   R   F   S   G   S   G   S   G   T   D
TTC ACT CTC ACC ATC AGC AGT CTG CAA CCT GAA GAT TTT GCT
F   T   L   T   I   S   S   L   Q   P   E   D   F   A
ACG TAC TAC TGT CAA CAG GTT CGG AAG GTG CCT CGG ACG TTC
T   Y   Y   C   Q   Q   V   R   K   V   P   R   T   F
GGC CAA GGG ACC AAG GTG GAA ATC AAA CGG
G   Q   G   T   K   V   E   I   K   R

```

FIG. 17

Inhibition biacore of MSA16

(a)

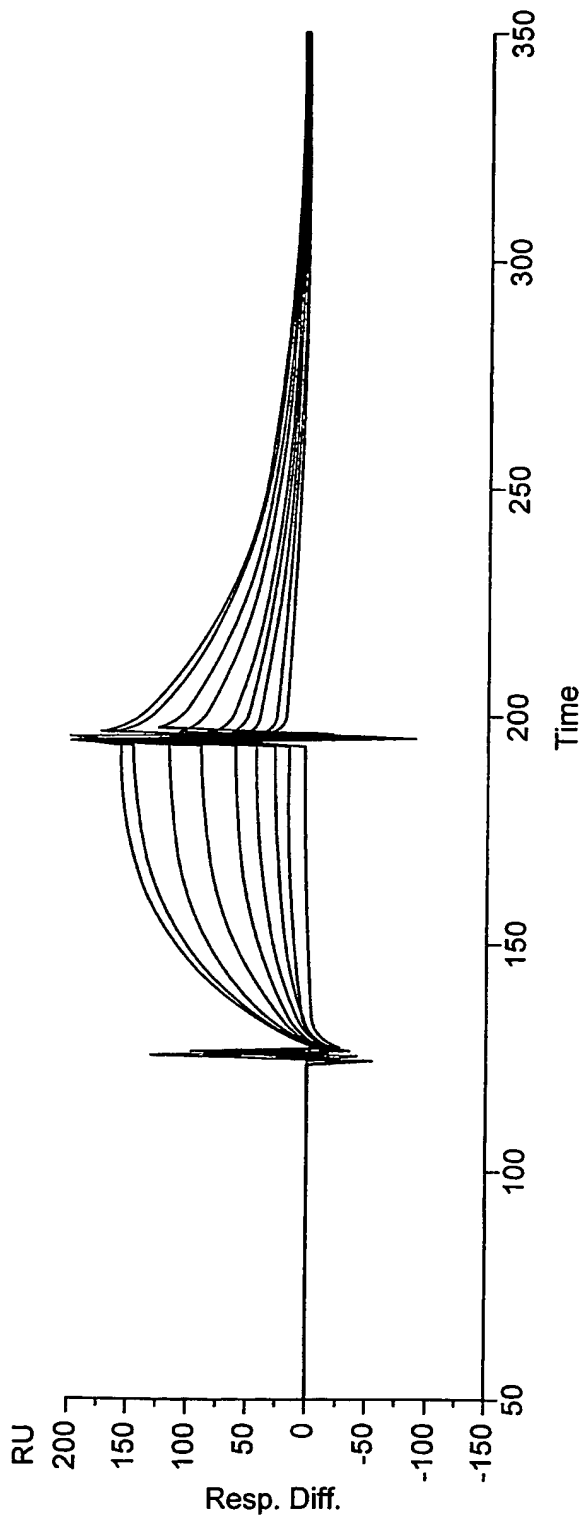
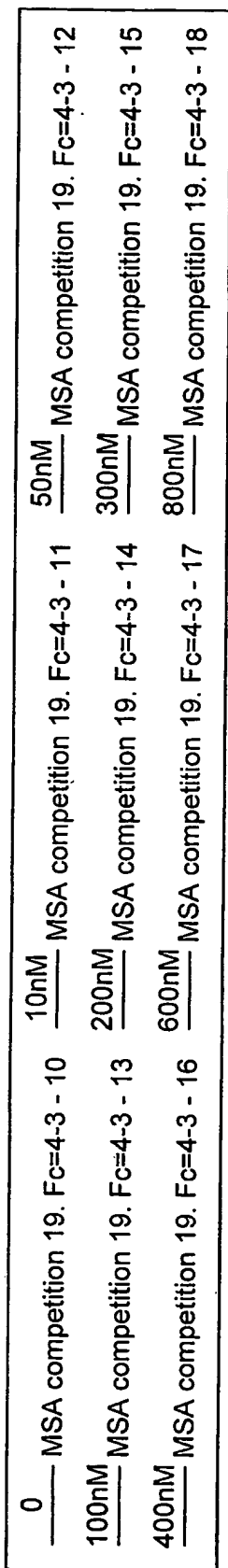
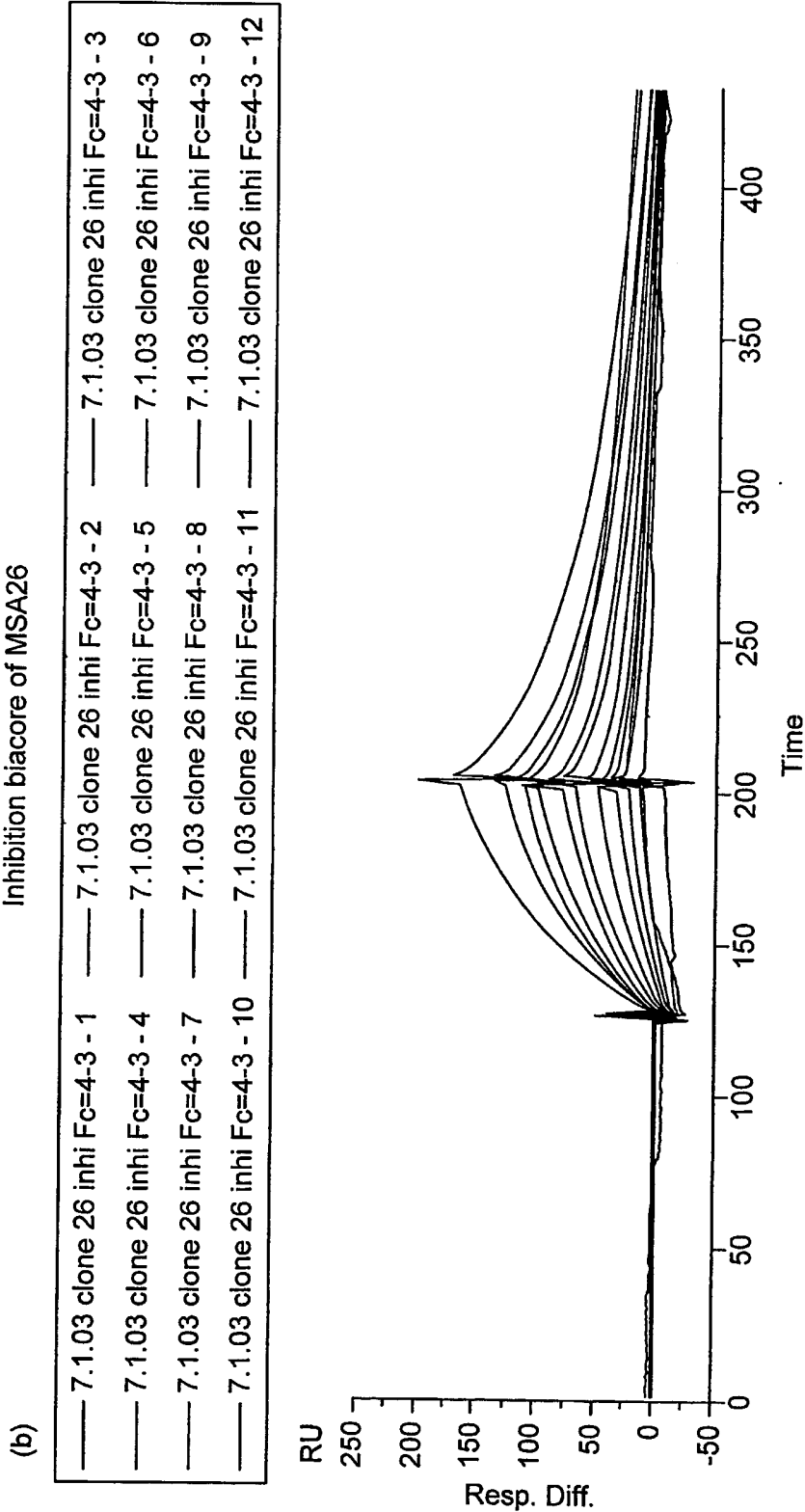


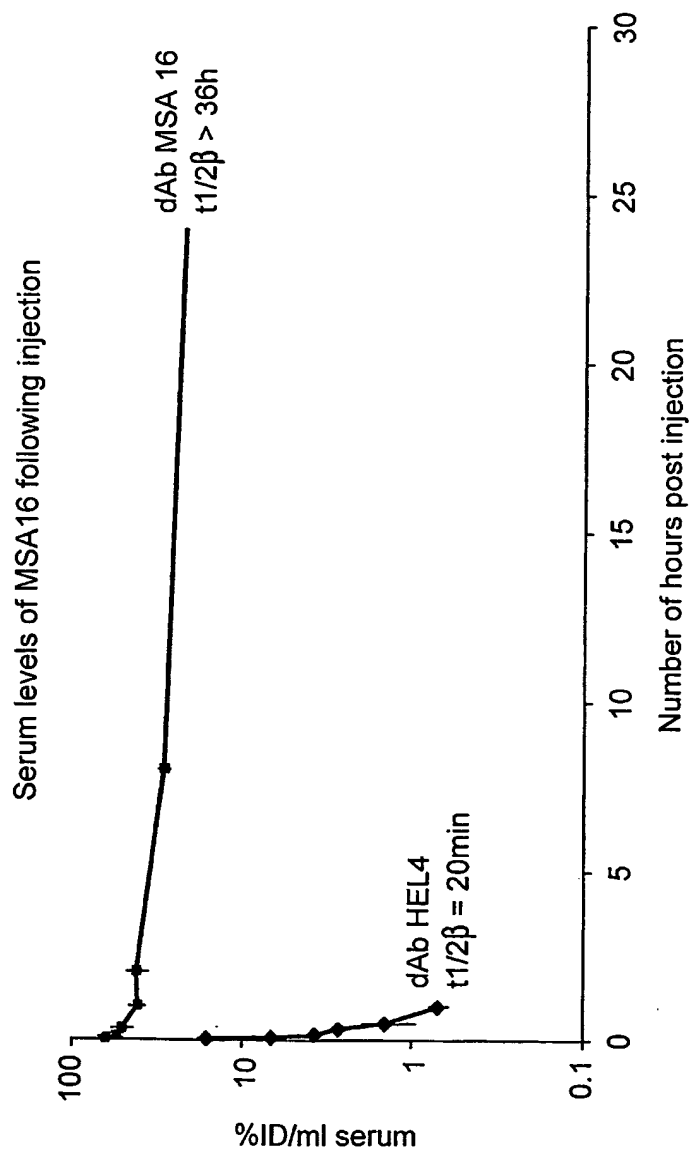
FIG. 17 CONT'D

Inhibition biacore of MSA26



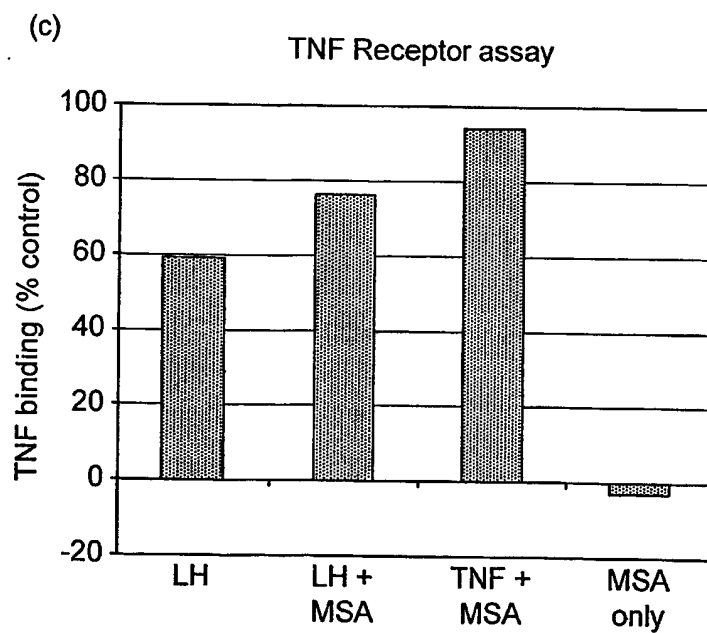
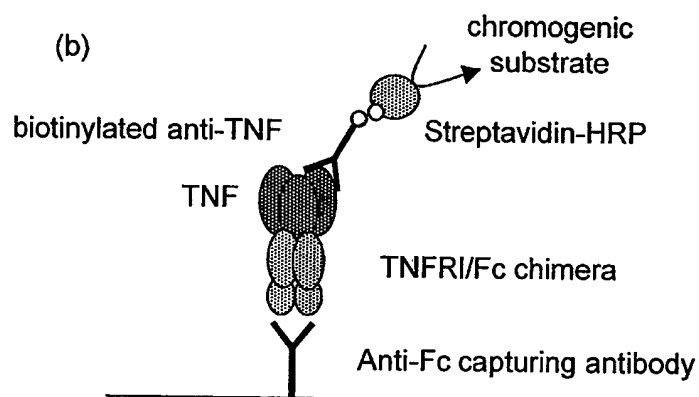
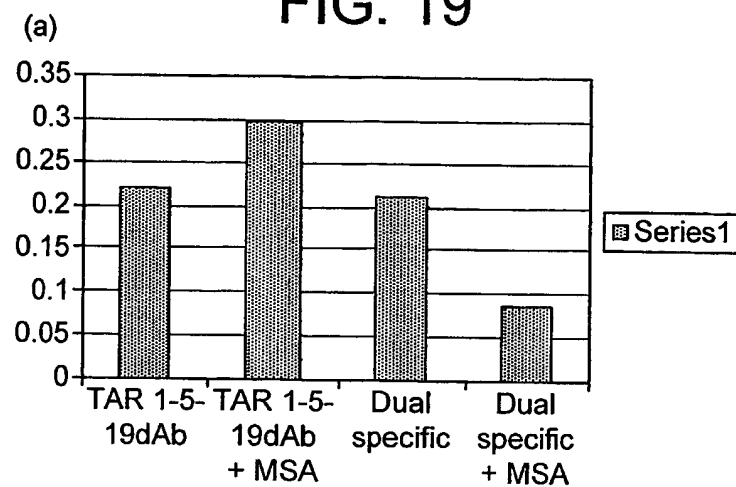
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FIG. 18



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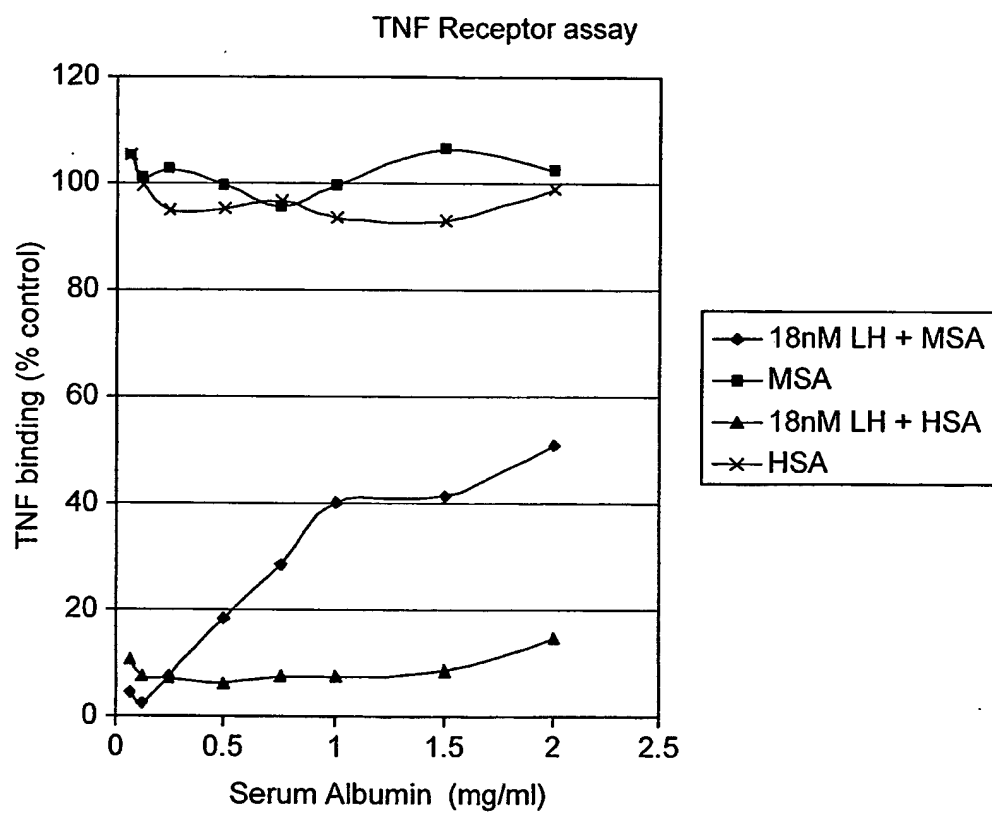
FIG. 19





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FIG. 20



## SEQUENCE LISTING

HEL4  
 5 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F R I S D E D M .  
 GAGGTGCAGC TGTGGAGTC TGGGGGAGGC TTGGTACAGC CTGGGGGCTC CCTGGTCTC TCCTGTGCAG CCTCCGGATT TAGGATTAGC GATGAGGATA  
 . G W V R Q A P G K G L E W V S S I Y G P S G S T Y Y A D S V K G R .  
 TGGGCTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTATCAAGC ATTATGSCC CTAGCGGTAG CACATACTAC GCAGACTCCG TGAAGGGCCG  
 10 . F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A S A L  
 GTTCACCATC TCCCGTGACA ATTCCAAGAA CACGCTGTAT CTGCATATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTATTGCGC GAGTGCCTTG  
 E P L S E P L G F W G Q G T L V T V S S  
 GAGCCGCTTT CGGAGCCCTT GGGCTTTTGG GGTCAAGGAA CCCTGTCTCAC CGTCTCGAGC  
 15  
 TAR2-5  
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F D L Y N M .  
 GAGGTGCAGC TGTGGAGTC TGGGGGAGGC TTGGTACAGC CTGGGGGCTC CCTGGTCTC TCCTGTGCAG CCTCCGGATT CACCTTTGAT CTTTATAATA  
 20 . F W V R Q A P G K G L E W V S F I S Q T G R L T W Y A D S V K G R .  
 TGTTTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCATTT ATTAGTCAGA CTGGTAGGCT TACATGCTAC GCAGACTCCG TGAAGGGCCG  
 . F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K T L  
 GTTCACCATC TCCCGTGACA ATTCCAAGAA CACGCTGTAT CTGCATATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAAACGCTG  
 25 E D F D Y W G Q G T L V T V S S  
 GAGGATTTTG ACTACTGGG CCAGGGAACC CTGGTCACCG TCTCGAGC  
 30  
 TAR1-5-19  
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S V K E F L W .  
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTCCC GGGCAAGTCA GAGCGTTAAG GAGTTTAT  
 35 . W Y Q Q K P G K A P K L L I Y M A S N L Q S G V P S R F S G S G S .  
 GGTGGTACCA GCAGAAACCA GGGAAAGCCC CTAGCTCCT GATCTATATG GCATCCAATT TGCAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC  
 . G T D F T L T I S S L Q P E D F A T Y Y C Q Q K F K L P R T F G Q  
 TGGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTAGCTACTA CTGTCAACAG AAGTTTAAAG TGCCTCGTAC GTTCGGCCAA  
 40 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG

TAR2-10  
 GAGGTGCAGCTGTTGGAGTCTGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTCGCTCTCTCTGTGCAGCCTCCGGATTACCTTTGAGTGGTATTGGATGGGTGGGTCCGC  
 CAGGCTCCAGGAAGGTTCTAGAGTGGGTCTCAGATTAGTGGTGGTGGTAGCACATACACGAGACTCCGTGAAGGCCGGTTCCATCTCCCGCGACAATTCCAAGA  
 ACACGCTGTATCTGCAATGAACAGCCTGCTGCGAGGACACCGGGTATATTACTGTGCGAAAGTTAAGTTGGGGGGGGCCCTAATTTTGTACTACTGGGGCCAGGAACCCCT  
 GGTACCCGTCTCGAGCGCEVQLLESGLVQPGSLRLSCAASGFTFEWYMWGVRQAPGKGLEWVSALSGSGSTYYADSVKGRFTISRDNKNTLYLQMNSLRATEDTAVYCA  
 KVKLGGGPNFDYWGQGLTVSS

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E V Q L L E S G G G L V Q P G G S .  
 1 GAGGTGCAGC TGTGGAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC  
 . L R L S C A A S G F T F E W Y W M .  
 51 CCTCGTCTC TCCTGTGCAG CCTCCGGATT CACCTTGAG TGGTATTGA  
 . G W V R Q A P G K G L E W V S A  
 101 TGGGTTGGGT CCGCCAGGCT CCAGGAAGG GTCTAGAGTG GGTCTCAGCT  
 I S G S G S T Y Y A D S V K G R .  
 151 ATTACTGGTA GTGGTGGTAG CACATACTAC GCAGACTCCG TGAAGGCCG  
 . F T I S R D N S K N T L Y L Q M N .  
 201 GTTCACCATC TCCCGGACA ATCCAGAA CACGCTGTAT CTGCAATGA  
 . S L R A E D T A V Y Y C A K V K  
 251 ACAGCTGCG TGCCGAGGAC ACCGCGTAT ATTACTGTGC GAAAGTTAAG  
 L G G G P N F D Y W G Q G T L V T .  
 301 TTGGGGGGG GGCCTAATT TGACTACTGG GGCAGGGA CCCTGCTCAC  
 Aval  
 -----  
 . V S S  
 351 CGTCTCGAGC

SUBSTITUTE SHEET (RULE 26)

30 TAR-1 dab DNA and amino acid sequences.  
 dabs 1, 2 and 3 are the partner sequences from TAR1-5 dimers 1-6. dab1 is the partner dab to dimers 1, 5 and 6  
 the second dabs are the same but the linker lengths are different, likewise, dab2 is the partner dab to dimers  
 2 and 3, dab3 is the partner dab to dimer 4. \* indicates the presence of an amber stop codon. Sequence homology  
 between, TAR1-5, TAR1-5-19 and the unique second dabs is 88%

35 TAR1-5-19  
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I D S Y L H  
 GACATCCAGA TGACCCAGTC TCCATCTCT CTGTCTCAT CTGTAGGAGA CCGTGCACC ATCACTGCC GGGCAAGTCA GAGCATTCAT AGTTATTAC  
 CTGTAGTCT ACTGGGTGAG AGGTAGGAGA GACAGAGTA GATGACCTCT GGCACACTGG TAGTGAACGG CCGTTCAGT CTGTACTA TCAATAATG  
 W Y Q Q K P G K A P K L L I Y S A S E L Q S G V P S R P S G S G S

40

ATTGGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGT GCATCCGAGT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC  
 TAACCATGGT CGTCTTTGGT CCCTTTCCGG GATTCGAGGA CTAGATATCA CGTAGCTCA AGCTTTCCAC CAGGGTAGT GCAAGTCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q V V W R P F T F G Q  
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAGATTTTG CTAGTACTA CTGTCAACAG GTTGTGGGC GTCCCTTTTAC GTTCGGCCNA  
 ACCCTGTCTA AAGTGAAGT GGTATGGTGC AGACGTTGGA CTCTTAAAC GATGCATGAT GACAGTTGTC CAACACACCG CAGGAAATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAG TGGAAATCAA ACGC  
 CCCTGGTCC ACCTTTAGTT TGCG

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TAR1-5  
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I P M N L L  
 GACATCCAGA TGACCCAGTC TCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCC ATCATTGCC GGGCAGTCA GAGCATTTT ATGATTTAT  
 CTGTAGTCT ACTGGTCAG AGGTAGGAGG GACAGAGGTA GACATCTCT GGCACATGG TAGTGAACGG CCCGTTTCA GTCTGTAATA TACTTAATA  
 W Y Q Q K P G K A P K L L I Y N A S V L Q S G V P S R F S G S G S  
 TGTGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGT GCATCCGAGT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC  
 ACACCATGTT CGTCTTTGGT CCCTTTCCGG GATTCGAGGA CTAGATATTA CGTAGGCACA ACGTTTCACC CCAGGTTAGT GCAAGTCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q V V W R P F T F G Q  
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAGATTTTG CTAGTACTA CTGTCAACAG GTTGTGGGC GTCCCTTTTAC GTTCGGCCNA  
 ACCCTGTCTA AAGTGAAGT GGTATGGTGC AGACGTTGGA CTCTTAAAC GATGCATGAT GACAGTTGTC CAACACACCG CAGGAAATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAG TGGAAATCAA ACGG  
 CCCTGGTCC ACCTTTAGTT TGCG

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Monomer - dabl: Partner dab in TAR1-5d1 (3ulinker), d5 (5U linker), d6 (7U linker)  
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I Y D A L B  
 GACATCCAGA TGACCCAGTC TCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCC ATCATTGCC GGGCAGTCA GAGCATTTT GATCGTTAG  
 CTGTAGTCT ACTGGTCAG AGGTAGGAGG GACAGAGGTA GACATCTCT GGCACATGG TAGTGAACGG CCCGTTTCA GTCTGTAATA CTACCAATC  
 W Y Q Q K P G K A P K L L I Y T A S R L Q S G V P S R F S G S G S  
 AGTGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGT GCATCCGAGT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC  
 TCACCATGTT CGTCTTTGGT CCCTTTCCGG GATTCGAGGA CTAGATATGA CGTAGGCACA ACGTTTCACC CCAGGTTAGT GCAAGTCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q V M Q R P V T F G Q  
 TGGACAGAT TTCACCTCTCA CCATCAGCAG TCTGCAACCT GAGATTTTG CTAGTACTA CTGTCAACAG GTTATGCAGC GTCCCTGTTAC GTTCGGCCNA  
 ACCCTGTCTA AAGTGAAGT GGTATGGTGC AGACGTTGGA CTCTTAAAC GATGCATGAT GACAGTTGTC CAGGAAATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAG TGGAAATCAA ACGG  
 CCCTGGTCC ACCTTTAGTT TGCG

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Monomer - dab2: Partner dab in TAR1-5d2 (3U linker), d3 (5U linker)  
 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I Y D A L \*  
 GACATCCAGA TGACCCAGTC TCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCC ATCATTGCC GGGCAGTCA GAGCATTTT GATCGTTTAC  
 CTGTAGTCT ACTGGTCAG AGGTAGGAGG GACAGAGGTA GACATCTCT GGCACATGG TAGTGAACGG CCCGTTTCA GTCTGTAATA CTACGAAATG  
 W Y Q Q K P G K A P K L L I Y T A S R L Q S G V P S R F S G S G S  
 AGTGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGT GCATCCGAGT TGCAAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC  
 TCACCATGTT CGTCTTTGGT CCCTTTCCGG GATTCGAGGA CTAGATATGA CGTAGGCACA ACGTTTCACC CCAGGTTAGT GCAAGTCAC CGTCACCTAG

45

G T D P T L T I S S L Q P E D F A T Y H C Q Q V M Q R P V T P G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTACGTACCA GTTATGCAAG GTTCCTGTAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAAGT GGTAGTCTC AGAGCTTGGG CTCTTAAAC GATGCATGTT GACAGTTGTC CAATACCTGC CAGGACAAATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTCC ACCTTAGTT TGCC

Monomer - dab3: Partner dab in TAB1-5d4 (5U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S V K E F L W  
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GAGCGTTAAG GAGTTTAT  
 CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCTCTT GGCACAGTGG TAGTGAACGG CCGTTTCACT CTGCAATTC CTCAAAATA  
 W Y Q Q K P G K A P K L L I Y M A S N L Q S G V P S R F S G S  
 GGTGTACCA GCGAAACCA GGGAAAGCCC CTAGCTCTCT GATCTATATG GCATCCAAAT TGCBAAGTGG GTTCCCATCA CGTTTCAGTG GCAGTGGATC  
 CCACCATGTT GGTCTTTGGT CCGTTTCGGG GATTGAGGA CTAGATATAC CGTAGGTAA ACCTTTTACC CCAAGGTAGT GCAAGTCAAC CGTCACCTAG  
 G T D P T L T I S S L Q P E D F A T Y Y C Q Q K F K L P R T F G Q  
 TGGGACAGT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTAGTACTA CTGTCAACAG AGTTTAAAG TGCCTGCTAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAAGT GGTAGTCTC AGACCTTGGG CTCTTAAAC GATGCATGAT GACAGTTGTC TTCAAAATTC ACGGAGCATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTCC ACCTTAGTT TGCC

TAB1-27 and related partner dab amino acid and DNA sequences (\* indicates the presences of an amber stop codon).  
 Sequence homology between TAB1-27 and the unique second dabs is 90.4%.

TAB1-27

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I W T K L H  
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GAGONTTGG ACGAAGTTAC  
 CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCTCTT GGCACAGTGG TAGTGAACGG CCGTTTCACT CTGCTTAAAC TGCTTCAATG  
 W Y Q Q K P G K A P K L L I Y M A S N L Q S G V P S R F S G S G S  
 ATGTGTACCA GCGAAACCA GGGAAAGCCC CTAGCTCTCT GATCTATATG GCATCCAAAT TGCBAAGTGG GTTCCCATCA CGTTTCAGTG GCAGTGGATC  
 TAACCATGTT CGTCTTTGGT CCGTTTCGGG GATTGAGGA CTAGATATAC CGTAGGTCAA ACCTTTTACC CCAAGGTAGT GCAAGTCAAC CGTCACCTAG  
 G T D P T L T I S S L Q P E D F A T Y Y C Q Q W F S N P S T F G Q  
 TGGGACAGT TTCACCTCA CCATCAGCAG TCTGCAACCT GAAGATTTTG CTAGTACTA CTGTCAACAG TGGTTTAAAG TGCCTGCTAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAAGT GGTAGTCTC AGACCTTGGG CTCTTAAAC GATGCATGAT GACAGTTGTC ACCAATCAT TAGCATCATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTCC ACCTTAGTT TGCG

Partner dab monomer in TAB1-27d1 (3U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I \* P I L C  
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GAGCATTTAG CCGATTTTAT  
 CTGTAGGTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCTCTT GGCACAGTGG TAGTGAACGG CCGTTTCACT CTGCTTAAAT GCGTAAATA  
 W Y Q Q K P G K A P K L L I Y A A S S L Q S G V P S R F S G S G S

GTTGTATACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATGCT GCATCCAGTT TGCRAAGTGG GGTCCCATCA CGTTTCAGTG GCAGTGGATC  
 CNAACATGGT CGTCTTTGGT CCCTTTCCGG GATTGAGGA CTAGATACGA CGTAGGTAGT CCAGGTTAGT GCAAAAGTAC CGTCAACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q I Q H I P V T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGG CTAGCTACTA CTGTCAACAG ATTCAGCATY TTCCCTGTGAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGTAGT GGTAGTCGTC AGACGTTGGA CTCTAAAC GATGCATGAT GACAGTTGTC TTAAGTCGTAT AAGGACACATG CAAAGCCGGT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTTAGTT TGCC

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Partner dAb monomer in TARI-27d2 (3U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I G \* D L H  
 GACATCCAGA TGACCCAGTC TCCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAAGTCA GAGCATTGGG TAGGATTATC  
 CTGTAGTCT ACTGGTCTAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCGCTTCAGT CTGCTAACCC ATCTAAATG  
 W Y Q Q K P G K A P K L L I Y T A S L L Q S G V P S R F S G S  
 ATTGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC  
 TAAACATGGT CGTCTTGGT CCCTTTCCGG GATTGAGGA CTAGATATGC CGTAGGAGAA ACGTTTCACC CCAGGTTAGT GCAAAAGTAC CGTCACTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q S A F P N T L G Q  
 TGGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGG CTAGCTACTA CTGTCAACAG CAGAGTGTCT TTCTTAATAC GCTCGGCCAA  
 ACCCTGTCTA AAGTGTAGT GGTAGTCGTC AGACGTTGGA CTCTAAAC GATGCATGAT GACAGTTGTC GTCTCAGAA AAGGATTATG CGAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTTAGTT TGCC

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Partner dAb monomer in TARI-27d7 (3U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I T K N L L  
 GACATCCAGA TGACCCAGTC TCCATCTCC CTGTCTGCAT CCGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAAGTCA GAGCATTGGG TAGGATTATC  
 CTGTAGTCT ACTGGTCTAG AGGTAGGAGG GACAGACGTA GGCATCTCT GGCACAGTGG TAGTGAACGG CCGCTTCAGT CTGCTAACCC ATCTTAATG  
 W Y Q Q K P G K A P K L L I Y \* A S L L Q S G V P S R F S G S  
 TTTGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC  
 AAACATGGT CGTCTTGGT CCCTTTCCGG GATTGAGGA CTAGATATGC CGTAGGAGAA ACGTTTCACC CCAGGTTAGT GCAAAAGTAC CGTCACTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q L R H K P P T F G Q  
 TGGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTGG CTAGCTACTA CTGTCAACAG CAGAGTGTCT TTCTTAATAC GCTCGGCCAA  
 ACCCTGTCTA AAGTGTAGT GGTAGTCGTC AGACGTTGGA CTCTAAAC GATGCATGAT GACAGTTGTC TCGAGGCTG CAAAGCCGGT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTTAGTT TGCC

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Partner dAb monomer in TARI-27d8 (3U linker)

D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I \* K S L R  
 GACATCCAGA TGACCCAGTC TCCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACCC ATCACTTGCC GGGCAAGTCA GAGCATTGGG TAGGATTATC  
 CTGTAGTCT ACTGGTCTAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCGCTTCAGT CTGCTAACCC ATCTTAATG  
 W Y Q Q K P G K A P K L L I Y H A S D L Q S G V P S R F S G S  
 GGTGTACCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC GATCTATAGC  
 CCACCATGGT CGTCTTGGT CCCTTTCCGG GATTGAGGA CTAGATATGC CGTAGGAGTAA ACGTTTCACC CCAGGTTAGT GCAAAAGTAC CGTCACTAG

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G T D F T L T I S S L Q P E D F A T Y Y C Q Q M V N S P V T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAGATTTTG CTAGTACTTA GTCTCAACAG ATGGTTAATA GTCTGTTTAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAAGT GGTAGTCTCT AGACGTTTGA CTCTTAAC GATGCATGAT GACAGTTGTC TACCAATTAT CAGGACAAATG CAAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTAGTT TGCC

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10 Partner dAb monomer in TARI-27d12 (3U linker)  
 D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I \* T A L H  
 GACATCCAGA TGACCCAGTC TCATCCTCC CTGTCTGCTAT CTGTAGGAGA CCGTGTACC ATCACTTGCC GGGCAAGTCA GAGCATTTAG ACGCGTTTAC  
 CTGTAGTCT ACTGGTCTAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCGTTTCAGT CTGCTAAATC TGCCGCAATG  
 W Y Q Q K P G K A P K L L I Y S A S L Q S G V P S R F S G S G S  
 ATTGTACCA GCAGAACCA GGGAAAGCC CTAAGTCTCT GATCTATCT GATCCAGTT TGCAGAGTGG GGTCCCATCA CGTTTCAGT GCAATGGATC  
 TRACCATGTT CGTCTTTGTT CCCTTTGGG GATTCGAGGA CTAGATAAGA CGTAGTCAA AGTTTCACC CCAGGAGTAGT GCAAGATCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q S S F L P T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAGATTTTG CTAGTACTTA GTCTCAACAG TCAGTTTIT TGCTTTTAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAAGT GGTAGTCTCT AGACGTTTGA CTCTTAAC GATGCATGAT GACAGTTGTC AGCTCAAAA ACGGAAATG CAAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTAGTT TGCC

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25 Partner dAb monomer in TARI-27d16 (3U linker)  
 D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I G P N L E  
 GACATCCAGA TGACCCAGTC TCATCCTCC CTGTCTGCTAT CTGTAGGAGA CCGTGTACC ATCACTTGCC GGGCAAGTCA GAGCATTTAG CCGATTTTAC  
 CTGTAGTCT ACTGGTCTAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCGTTTCAGT CTGCTAAATC GGCTTAATC  
 W Y Q Q K P G K A P K L L I Y A A S L Q S G V P S R F S G S G S  
 AGTGTACCA GCAGAACCA GGGAAAGCC CTAAGTCTCT GATCTATCT GATCCAGTT TGCAGAGTGG GGTCCCATCA CGTTTCAGT GCAATGGATC  
 TCACCATGTT CGTCTTTGTT CCCTTTGGG GATTCGAGGA CTAGATAAGA CGTAGTCAA AGTTTCACC CCAGGAGTAGT GCAAGATCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q Q M G R P R T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAGATTTTG CTAGTACTTA GTCTCAACAG GATGTGGGC GTCTCGGAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAAGT GGTAGTCTCT AGACGTTTGA CTCTTAAC GATGCATGAT GACAGTTGTC AGCTCAAAA ACGGAAATG CAAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTAGTT TGCC

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35 Partner dAb monomer in TARI-27d23 (5U linker)  
 D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I K H \* L A  
 GACATCCAGA TGACCCAGTC TCATCCTCC CTGTCTGCTAT CTGTAGGAGA CCGTGTACC ATCACTTGCC GGGCAAGTCA GAGCATTTAG CATTAGTTAG  
 CTGTAGTCT ACTGGTCTAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACAGTGG TAGTGAACGG CCGTTTCAGT CTGCTAAATC GTATCAATC  
 W Y Q Q K P G K A P K L L I Y K A S V L Q S G V P S R F S G S G S  
 CTGTGTACCA GCAGAACCA GGGAAAGCC CTAAGTCTCT GATCTATCT GATCCAGTT TGCAGAGTGG GGTCCCATCA CGTTTCAGT GCAATGGATC  
 GACCATGTT CGTCTTTGTT CCCTTTGGG GATTCGAGGA CTAGATAAGA CCGTTTCACC CCAGGAGTAGT GCAAGATCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q L R R R P T T F G Q

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TGGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTCG CTAGCTACTA CTGTCAACAG CTTAGGGTC GTCTACTAC GTTGGGCCAA  
 ACCCTCTCTA AGTGTAGAGT GGTAGTCGTC AGACGTTGGA CTTCTAAAC GATGCATGAT GACAGTTGTC GATCCGCGAG CAGGATGATG CAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGNAATCAA ACGG  
 CCCTGGTTCC ACCTTTAGTT TGCC

Partner dAb monomer in TAB1-27d30 (7U linker)

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S V K A \* L T  
 GACATCCAGA TGACCCAGTC TCCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCACTGCC GGGCAAGTCA GAGGTTAAG GCTTAGTTAA  
 CTGTAGTCT ACTGGGTCAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACATGG TAGTGAAGG CCGTTTCAGT CTCGCAATTC CGAATCAATT  
 W Y Q Q K P G K A P K L L I Y K A S T L Q S G V P S R F S G S G S  
 CTTGGTACCA GCAGAACCA GGGAAAGCC CTAAGCTCT GATCTATAAG GCATCCACTT TGCAAGTGG GGTCCCATCA CGTTTCAGT GCAGTGGATC  
 GAACCATGGT CGTCTTTGGT CCCTTCGGG GATTCGAGGA CTAGATATTC CGTAGGTGAA ACGTTTCACC CCAGGGTAGT GCAAGTGCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q H S R P Y T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTCG CTAGCTACTA CTGTCAACAG CATAGTTTCTA GGCCTTATAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTTCTAAAC GATGCATGAT GACAGTTGTC GTATCAAGAT CCGGATATATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGNAATCAA ACGG  
 CCCTGGTTCC ACCTTTAGTT TGCC

Partner dAb monomer in TAB1-27d31 (7U linker)

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I E N R L G  
 GACATCCAGA TGACCCAGTC TCCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCACTGCC GGGCAAGTCA GAGGTTAAG GCTTAGTTAG  
 CTGTAGTCT ACTGGGTCAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACATGG TAGTGAAGG CCGTTTCAGT CTCGTAATC TTAGCCCAATC  
 W Y Q Q K P G K A P K L L I Y \* A S L L Q S G V P S R F S G S G S  
 GTTGGTACCA GCAGAACCA GGGAAAGCC CTAAGCTCT GATCTATAAG GCGTCTCTGT TGCAAGTGG GGTCCCATCA CGTTTCAGT GCAGTGGATC  
 CAACCATGGT CGTCTTTGGT CCCTTCGGG GATTCGAGGA CTAGATATTC CGCAGGAACA ACGTTTCACC CCAGGGTAGT GCAAGTGCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q D S Y F P R T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTCG CTAGCTACTA CTGTCAACAG GATTCGTATT TTCTCTGAT GTTCGGCCAA  
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTTCTAAAC GATGCATGAT GACAGTTGTC CTAAGCATAA AAGGAGCATG CAAGCCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGNAATCAA ACGG  
 CCCTGGTTCC ACCTTTAGTT TGCC

Partner dAb monomer in TAB1-27d36 (7U linker)

D I Q M T Q S P S L S A S V G D R V T I T C R A S Q S I M D K L K  
 GACATCCAGA TGACCCAGTC TCCATCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTACC ATCACTGCC GGGCAAGTCA GAGGTTAAG GCTTAGTTAA  
 CTGTAGTCT ACTGGGTCAG AGGTAGGAGG GACAGACGTA GACATCTCT GGCACATGG TAGTGAAGG CCGTTTCAGT CTCGTATATC CTATTCATTT  
 W Y Q Q K P G K A P K L L I Y \* A S I L Q S G V P S R F S G S G S  
 AGTGGTACCA GCAGAACCA GGGAAAGCC CTAAGCTCT GATCTATAAG GCATCCACTT TGCAAGTGG GGTCCCATCA CGTTTCAGT GCAGTGGATC  
 TCACCATGGT CGTCTTTGGT CCCTTCGGG GATTCGAGGA CTAGATATTC CGTAGGTAAA ACGTTTCACC CCAGGGTAGT GCAAGTGCAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q D S G G P N T F G Q



TGGGACGAGT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTACGTACTA CTGTCAACAG GATAGTGGG GTCCTAATAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTTCATAAAC GATGCATGAT GACATTCGAT CAGGATTAATG CAGGCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTAGTT TGCC

Partner ddb monomer in TAR1-27d37 (7U linker)

10 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I G R N L E  
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GACATTTAGG AGGAATTTAG  
 CTGTAGGTTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCGTTTCAGT CTGCTAACCC TCCTTAATC  
 W Y Q Q K P G K A P K L L I Y D A S H L Q S G V P S R F S G S G S  
 AGTGTACCA GCAGAAACCA GGGAAAGCC CTAAGTCTCT GATCTATGAT GCATCCCAT TGCMAAGTGG GGTCCCATCA CGTTTCAGTG GCATGTGATC  
 TCACATGGT CGTCTTTGGT CCCTTTGGG GATTCGAGGA CTGATPACTA CSTRAGGTAA ACCTTTCACC CCAGGGTAGT GCMAAGTCAAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q S R W L P R T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTACGTACTA CTGTCAACAG TCGCGTTGGC TTCTCGTAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTTCATAAAC GATGCATGAT GACATTCGAT CAGGATTAATG CAGGCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTAGTT TGCC

Partner ddb monomer in TAR1-27d39 (7U linker)

25 D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q S I R K M L V  
 GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGAGA CCGTGTCAAC ATCACTTGCC GGGCAAGTCA GACATTTAGG AAGATGTTAG  
 CTGTAGGTTCT ACTGGGTGAG AGGTAGGAGG GACAGACGTA GACATCCTCT GGCACAGTGG TAGTGAACGG CCGTTTCAGT CTGCTAACCC TTCTACAATC  
 W Y Q Q K P G K A P K L L I Y R A S Y L Q S G V P S R F S G S G S  
 TTGTGTACCA GCAGAAACCA GGGAAAGCC CTAAGTCTCT GATCTATGAT GCATCCCAT TGCMAAGTGG GGTCCCATCA CGTTTCAGTG GCATGTGATC  
 AAACCATGGT CGTCTTTGGT CCCTTTGGG GATTCGAGGA CTGATPAGCC CSTRAGGTAA ACCTTTCACC CCAGGGTAGT GCMAAGTCAAC CGTCACCTAG  
 G T D F T L T I S S L Q P E D F A T Y Y C Q Q A F R R P R T F G Q  
 TGGACAGAT TTCACTCTCA CCATCAGCAG TCTGCAACCT GAAGATTTC CTACGTACTA CTGTCAACAG GCTTTTGGC GGCCTAGGAC GTTCGGCCAA  
 ACCCTGTCTA AAGTGAGAGT GGTAGTCGTC AGACGTTGGA CTTCATAAAC GATGCATGAT GACATTCGAT CAGGATTAATG CAGGCGGTT  
 G T K V E I K R  
 GGGACCAAGG TGGAAATCAA ACGG  
 CCCTGGTTCC ACCTTAGTT TGCC

40 TAR2 ddb amino acid and DNA sequences (\* indicates the presences of an amber stop codon) sequence homology  
 between TAR2h-5 and the unique second dabs is 83.5%

TAR2h-5

45 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F D L Y N M  
 GAGGTGAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGGTC CTTGGTCTC TCCTGTGTCG CTTGGGATTT CACCTTTGAT CTTTATATA  
 CTCCAGTGG ACACCTCAG ACCCTCTCG AACCATGTG AACCATGTG GACCCCTCG GAGCCAGAG AGGACAGTC GTGGGCTAA GAAATATAT  
 F W V R Q A P G K G L E W V S F I S Q T G R L T W Y A D S V K G R

TGTCTTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCATTT ATTATGTCAGA CTGGTAGGCT TACATGGTAC GCAGACTCCG TGAAGGGCCG  
 ACANAACCA GCGGTCCTCA GGTCCCTTCC CAGATCTCAC CCAGAGTAA TAATCATGCT GACCATCCGA ATGTACCATG CGTCTGAGGC ACTTCCCGGC  
 P T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K T L  
 GTTCACATC TCCCGGACA ATTCCAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGTAT ATTACTGTGC GAAACGCTG  
 CAGTGTAG AGGGCGCTGT TAAGGTTCT GTGCAATGA GAGTTTACT TGTGGACGC ACGGCTCTG TGGCGCCATA TAATGACACG CTTTTCGAC  
 E D F D Y W G Q G T L V T V S  
 GAGATTG ACTACTGGG CCGAGGAACC GTGTCACCG TCTCG  
 CTCCTAAAC TGATGACCC GGTCCCTTGG CACAGTGGC AGAGC

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Partner dAb monomer in TAR2h-5d1 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T P P V Y M M  
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGTC CCTGCTCTC TCCTGTGCG CTTCCGGATT CACCTTTCCG GTTTATATGA  
 CTCACGTG ACACCTCAG ACCCCCTCCG AACATGTG GACCCCCAG GGAGCGAG AGGACAGTC GGAGGCTTAA GTGGAAGGC CAATATAT  
 G W V R Q A P G K G L E W V S S I D A L G G R T G Y A D S V K G R  
 TGGTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCATG ATTGATGCT TTGGTGGCG GCAGCTTAC GCAGACTCCG TGAAGGGCCG  
 ACCRAACCA GCGGTCGA GTTCCCTTCC CAGATCTCAC CCAGAGTAGC TAACTACGAG AACCAACCGC CTGTCCATG CGTCTGAGC ACTTCCCGGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K T M  
 GTACATC TCCCGGACA ATTCCAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGTAT ATTACTGTGC GAAACTATG  
 CAGTGTAG AGGGCGCTGT TAAGGTTCT GTGCAATGA GAGTTTACT TGTGGACGC ACGGCTCTG TGGCGCCATA TAATGACACG CTTTGTATAC  
 S N K T H T F D Y W G Q G T L V T V S  
 TGAATAAGA CGATACGTT TGACTACTGG GGCCAGGAA CCTGTGTAC CGTCTCG  
 AGCTTATCT GGTATGCAA ACTGATGACC CCGTCCCTT GGGACCAAGT GCAGAGC

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Partner dAb monomer in TAR2h-5d2 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T P V A Y N M  
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGTC CCTGCTCTC TCCTGTGCG CTTCCGGATT CACCTTTCCG GTTTATATGA  
 CTCACGTG ACACCTCAG ACCCCCTCCG AACATGTG GACCCCCAG GGAGCGAG AGGACAGTC GGAGGCTTAA GTGGAAGGC CAATATAT  
 T W V R Q A P G K G L E W V S S I N T F G N \* T R Y A D S V K G R  
 TGACTGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCATG ATTGATGCT TTGGTGGCG GCAGCTTAC GCAGACTCCG TGAAGGGCCG  
 ACTGAACCA GCGGTCGA GTTCCCTTCC CAGATCTCAC CCAGAGTAGC TAAATATGA AACCATTAAT CTGTTCATG CGTCTGAGC ACTTCCCGGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K G S  
 GTTCACATC TCCCGGACA ATTCCAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGTAT ATTACTGTGC GAAAGGTAGT  
 CAGTGTAG AGGGCGCTGT TAAGGTTCT GTGCAATGA GAGTTTACT TGTGGACGC ACGGCTCTG TGGCGCCATA TAATGACACG CTTTCCATCA  
 R P F D Y W G Q G T L V T V S  
 AGCCCTTGG ACTACTGGG CCGAGGAACC CTGTACCG TCTCG  
 TCCGAAAAC TGATGACCC GGTCCCTTGG GACAGTGGC AGAGC

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Partner dAb monomer in TAR2h-5d3 (3U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T P \* G Y R M  
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGTC CCTGCTCTC TCCTGTGCG CTTCCGGATT CACCTTTCCG GGTATATGA  
 CTCACGTG ACACCTCAG ACCCCCTCCG AACATGTG GACCCCCAG GGAGCGAG AGGACAGTC GGAGGCTTAA GTGGAAGGC CAATATAT  
 G W V R Q A P G K G L E W V S W I T R T G G T T Q Y A D S V K G R  
 TGGTGGGT CCGCCAGGCT CCAGGGAAGG GTCTAGAGTG GGTCTCATG ATTACGGTA CTGTGGGAC GACACAGTAC GCAGACTCCG TGAAGGGCCG  
 ACCRAACCA GCGGTCGA GGTCCCTTCC CAGATCTCAC CCAGAGTAGC TAAATGCAAT GACCCCTG CTGTGTCTG CGTCTGAGC ACTTCCCGGC

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P T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K P A  
 GTTCACATC TCCGCGACA ATTCCAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAACCGGCG  
 CAACTGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GACTTACT TGTGCGAGC ACAGCTCTG TGCGGCCATA TATGACACG CTTTGGCCG  
 K L V G V G F D Y W G Q G T L V T V S  
 AAGCTGTG GGTGGGT TGAATCTG GGCAGGAA CCTGTGTAC CGTCTCG  
 TTCGACAC CCACCCAA ACTGATGACC CCGTCCCTT GGCACGAGT GCGAGC

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Partner dAb monomer in TAR2h-5d4 (3U linker)  
 E V Q L L E S G G L V Q P G G S L R L S C A A S G F T F R K Y \* M  
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCAG CTCCCGGATT CACCTTTCG AGTATTAGA  
 CTCCAGTCG ACACCTCAG ACCCTCCG AACCATGTCG GACCCCCAG GAGCGAGAG AGACACATC GGAGGCTAA GTGGAAGCC TTCATATCT  
 G W V R Q A P G K G L E W V S Q I G A K G Q S T D Y A D S V K G R  
 TGGGTGGGT CGCCAGGCT CCAGGAGG GTCTAGAGTG GGTCTCAG ATTGTGCGA AGGTCTATC TACAGATTAC GCAGACTCCG TGAAGGCGG  
 ACCCACCA GCGGTCCGA GTCCTCTCC CAGATCTCAG CCAGAGTGT TAACACGCT TCCAGTCAG ATGTCTAATG CGTCTGAGC ACTTCCGCG  
 P T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K K K  
 GTTACCATC TCCCGGACA ATTCAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAGAAGAG  
 CAACTGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GAGTTTACT TGTGCGAGC ACAGCTCTG TGCGGCCATA TATGACACG CTTTCTCTC  
 R G E N Y F F D Y W G Q G T L V T V S  
 AGGGGGAGA ATTATTTT TGAATCTG GGCAGGAA CCTGTGTAC CGTCTCG  
 TCCCCCTCT TAATAAANA ACTGATGACC CCGTCCCTT GGCACGAGT GCGAGC

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Partner dAb monomer in TAR2h-5d5 (3U linker)  
 E V Q L L E S G G L V Q P G G S L R L S C A A S G F T F R K Y S M  
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCAG CTCCCGGATT CACCTTTCG CGGTATAGA  
 CTCCAGTCG ACACCTCAG ACCCTCCG AACCATGTCG GACCCCCAG GAGCGAGAG AGACACATC GGAGGCTAA GTGGAAGCC GCCATATCAT  
 S W V R Q A P G K G L E W V S D I S R S G R Y T H Y A D S V K G R  
 TGTGTGGGT CGCCAGGCT CCAGGAGG GTCTAGAGTG GGTCTCAGAT ATTCTCGT CTGGTGGTA TACACATTAC GCAGACTCCG TGAAGGCGG  
 ACAGCACCA GCGGTCCGA GTCCTCTCC CAGATCTCAG CCAGATCTA TAAAGACAA GACAGCCAT ATGTCTAATG CGTCTGAGC ACTTCCGCG  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K R I  
 GTTACCATC TCCCGGACA ATTCAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAACGTATT  
 CAACTGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GAGTTTACT TGTGCGAGC ACAGCTCTG TGCGGCCATA TATGACACG CTTTGGCATA  
 D S S Q N G F D Y W G Q G T L V T V S  
 GATTCCTCT AGATGGGT TGAATCTG GGCAGGAA CCTGTGTAC CGTCTCG  
 CTAAGAAG TCTTACCA ACTGATGACC CCGTCCCTT GGCACGAGT GCGAGC

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Partner dAb monomer in TAR2h-5d6 (3U linker)  
 E V Q L L E S G G L V Q P G G S L R L S C A A S G F T F \* G Y K M  
 GAGTGCAGC TGTGGAGTC TGGGGAGGC TTGGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCAG CTCCCGGATT CACCTTTCG GGTATAGA  
 CTCCAGTCG ACACCTCAG ACCCTCCG AACCATGTCG GACCCCCAG GAGCGAGAG AGACACATC GGAGGCTAA GTGGAAGCC CCCATATCT  
 F W V R Q A P G K G L E W V S A I S G S G G T Y Y A D S V K G R  
 TGTGTGGGT CGCCAGGCT CCAGGAGG GTCTAGAGTG GGTCTCAGAT ATTACTGTG TGAAGGCGG  
 ACACACCA GCGGTCCGA GTCCTCTCC CAGATCTCAG CCAGATCTA TAAAGACAA GACAGCCAT ATGTCTAATG CGTCTGAGC ACTTCCGCG

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F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K Q K  
 GTTCACCATC TCCGCGACA ATTCNAGAA CACGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGGGTAT ATTACTGTGC GAACAGAAAG  
 CAAGTGGTAG AGGCGCTGT TAAGTTCTT TAAGTTCTT GTGCGACATA GACGTTTACT TGTGCGAGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTGTCTTC  
 E N F D Y W G Q G T L V T V S  
 GAGATTITG ACTACTGGG CCGAGGAACC CTGGTCACCG TCTCG  
 CTCCTAAAC TGATGACCCC GGTCCCTTGG GACCACTGGC AGAGC

Partner dab monomer in TAR2h-5d7 (3U linker)  
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F G D Y A M  
 GAGGTGACG TGTGTGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCG CCTCGGATT CACCTTTGGG GATTATGCTA  
 CTCACGTCG ACAACCTCAG ACCCCCTCG AACCATGTG GACCCCCAG GAGCGAGAG AGGACAGTC GAGGCGCTAA GTGGAACCC CTAATACGAT  
 W V R Q A P G K G L E W V S V I S S N G S T F Y A D S V K G R  
 TGTGTGGGT CCGCCAGGCT CCGAGGAGG GTCTAGAGTG GGTCTCAGTG ATTAGTTCGA ATGGTGGAG TACATTTAC GCAGACTCCG TGAAGGCGCG  
 ACACACCCA GGGGTCCGA GGTCCCTTCC CAGATCTCAG CAGAGTCTAC CCAGAGTCTC TAATCAAGCT TACCACCTC AIGTAAATG CGTCTGAGG ACTTCCCGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K R V  
 GTTCACCATC TCCGCGACA ATTCNAGAA CACGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGGGTAT ATTACTGTGC GAACAGTGT  
 CAAGTGGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GACGTTTACT TGTGCGAGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTGCACAA  
 R K R T P E F D Y W G Q G T L V T V S  
 COTAGAGGA CTCCTGAGTT TGACTACTGG GGCAGGGA CCTGTGTCAC CGTCTCG  
 GCATTTCTCT GAGGACTCAA ACTGATGACC CCGTCCCTT GGGACCACTG GCAGAGC

Partner dab monomer in TAR2h-5d8 (3U linker)  
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F R R Y K M  
 GAGGTGACG TGTGTGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCG CCTCGGATT CACCTTTAGG AGGTATAAGA  
 CTCACGTCG ACAACCTCAG ACCCCCTCG AACCATGTG GACCCCCAG GAGCGAGAG AGGACAGTC T N Y A D S V K G R  
 G W V R Q A P G K G L E W V S A I G R N G T K T N Y A D S V K G R  
 TGGTGTGGT CCGCCAGGCT CCGAGGAGG GTCTAGAGTG GGTCTCAGTG ATGGGAGGA ATGGTACGA GACAAATAC GCAGACTCCG TGAAGGCGCG  
 ACCCAACCA GGGGTCCGA GGTCCCTTCC CAGATCTCAG CAGAGTCTC TAACCTCTCT TACCATGCTT CTTTAAATG CGTCTGAGG ACTTCCCGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K I Y  
 GTTCACCATC TCCGCGACA ATTCNAGAA CACGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGGGTAT ATTACTGTGC GAACATTTAT  
 CAAGTGGTAG AGGCGCTGT TAAGTTCTT GTGCGACATA GACGTTTACT TGTGCGAGCG ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTAAATA  
 T G K P A A F D Y W G Q G T L V T V S  
 ACGGGGAGC CTGCTCGTT TGACTACTGG GGCAGGGA CCTGTGTCAC CGTCTCG  
 TGCCCTCTCG GAGGACGCAA ACTGATGACC CCGTCCCTT GGGACCACTG GCAGAGC

Partner dab monomer in TAR2h-5d9 (3U linker)  
 E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F K K Y \* M  
 GAGGTGACG TGTGTGAGTC TGGGGGAGC TTGGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCG CCTCGGATT CACCTTTAGG AGGTATTAGA  
 CTCACGTCG ACAACCTCAG ACCCCCTCG AACCATGTG GACCCCCAG GAGCGAGAG AGGACAGTC GAGGCGCTAA GTGGAATTC TTGATAATCT  
 S W V R Q A P G K G L E W V S A I S G S G S T Y Y A D S V K G R  
 TGTCTTGGT CCGCCAGGCT CCGAGGAGG GTCTAGAGTG GGTCTCAGTG ATTAGTGTGA GTGGTGTAG CACATATAC GCAGACTCCG TGAAGGCGCG  
 ACAGAACCA GGGGTCCGA GGTCCCTTCC CAGATCTCAG CAGATCTC TAATCACCAT CACCACCATC GTGTATGATG CGTCTGAGG ACTTCCCGC

F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K M L  
 GTTCACCATC TCCCGCGACA ATTCACAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAATGCTG  
 CAACTGGTAG AGGGGCTGT TAAGGTCTT GTGCGACATA GAGCTTACT TGTGCGACG ACGGCTCTG TGCGGCCATA TAATGACACG CTTTACGAC  
 R T K N K V F D Y W G Q G T L V T V S  
 AGACTAGAA ATAAAGTGT TGAATCTGG GGCAGGAA CCTGGTCA CGTCTG  
 TCCGTATCT TATTCACAA ACTGATGACC CCGTCCCTT GGCACCAATG GCGAGC

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10 Partner dAb monomer in TAR2h-5d10 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F R R Y K M  
 GAGTGACG TGTGGAGTC TGGGGAGGC TTGTACAGC CTGCGGTCTC TCCTGTGCG CCTCGGAT CACCTTTAGG AGGTATAAGA  
 CTCACGTCG ACACCTCAG ACCCCCTCCG AACNCTCG GAGCCCGAG AGGACAGTC GGAGGCTTAA GTGGAATCC TCCATATCT  
 G W V R Q A P G K G L E W V S I G R N G T K T N Y A D S V K G R  
 TGGTGTGGT CCGCCAGGCT CAGGGAAGG GTCTAGAGTG GGTCTCAGC ATTGGGAGGA ATGCTACGAA GACAAATTAC GCAGACTCG TGAAGGGCG  
 ACCCAACCA GCGGTCCGA GTCCCTTCC CAGNCTCAC CCAGATCGC TAACCTCTT TACCATGCTT CTGTTTAAG CGTCTGAGC ACTTCCCGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K I Y  
 GTTCACCATC TCCCGCGACA ATTCACAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAATTTAT  
 CAACTGGTAG AGGGGCTGT TAAGGTCTT GTGCGACATA GAGCTTACT TGTGCGACG ACGGCTCTG TGCGGCCATA TAATGACACG CTTTAAATA  
 T G K P A A F D Y W G Q G T L V T V S  
 ACGGGAAGC CTGCTGCTT TGAATGACC CCGTCCCTT GGCACCAATG GCGAGC

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25 Partner dAb monomer in TAR2h-5d11 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F \* S Y R M  
 GAGTGACG TGTGGAGTC TGGGGAGGC TTGTACAGC CTGCGGTCTC TCCTGTGCG CCTCGGAT CACCTTTAG AGTTATCGA  
 CTCACGTCG ACACCTCAG ACCCCCTCCG AACATCTCG GAGCCCGAG AGGACAGTC GGAGGCTTAA GTGGAATCC TCAATAGCT  
 G W V R Q A P G K G L E W V S I S R G R H T S Y A D S V K G R  
 TGGTGTGGT CCGCCAGGCT CAGGGAAGG GTCTAGAGTG GGTCTCAGT ATTCTCGA GGGGTAGGA TACATCTTAC GCAGACTCG TGAAGGGCG  
 ACCCAACCA GCGGTCCGA GTTCCCTTCC CAGATCTCAC CCAGATCTCA TAAGCTCA CCCCCTCGT ATGTAGATG CGTCTGAGC ACTTCCCGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K R V  
 GTTCACCATC TCCCGCGACA ATTCACAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCCGAGGAC ACCGCGGTAT ATTACTGTGC GAAAGGTT  
 CAACTGGTAG AGGGGCTGT TAAGGTCTT GTGCGACATA GAGCTTACT TGTGCGACG ACGGCTCTG TGCGGCCATA TAATGACACG CTTTCCCA  
 P G R G R S F D Y W G Q G T L V T V S  
 CCGGTGCGG GCGTCTTCTT TGAATGACC CCGTCCCTT GGCACCAATG GCGAGC

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40 Partner dAb monomer in TAR2h-5d12 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F P F R R Y R M  
 GAGTGACG TGTGGAGTC TGGGGAGGC TTGTACAGC CTGCGGTCTC TCCTGTGCG CCTCGGAT CACCTTTAG CCGTATCGA  
 CTCACGTCG ACACCTCAG ACCCCCTCCG AACATCTCG GAGCCCGAG AGGACAGTC GGAGGCTTAA GTGGAATCC TCAATAGCT  
 R W V R Q A P G K G L E W V S I S P G G K H T T Y A D S V K G R  
 TGAAGTGGT CCGCCAGGCT CAGGGAAGG GTCTAGAGTG GTCTCAGT ATTCTCGG GTGTTAGCA TACAACGTAC GCAGACTCG TGAAGGGCG  
 ACTCCACCA GCGGTCCGA GTTCCCTTCC CAGATCTCAC CCAGATCTCA TAAGAGGCC CACCATCTG ATGTTGATG CGTCTGAGC ACTTCCCGC

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F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K G E  
 GTTCCATC TCCCGGACA ATTCCAAGAA CACGCTGTAT CTGCAATGA ACAGCTGCG TGCAGGAC ACCGCGGTAT ATTACTGTGC GAAAGGTGAG  
 CNAAGTGTAG AGGGCGCTGT TAAGTTCTT GTGCGACATA GAGTTTACT TGTGCGACGC ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTCCACTC  
 G G A S S A F D Y W G Q G T L V T V S  
 GGGGGGCGA GTTCTGCTT TGACTACTGG GGCAGGGA CCTGTGTCAC CGTCTCG  
 CCCCCCGCT CAGACGCA ACTGATGACC CCGTCCCTT GGGACCAATG GCAGAGC

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10 Partner dab monomer in TAR2h-5d13 (5U linker)

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F \* R Y G M  
 GAGGTGAGC TGTGAGTC TGGGGAGGC TTGTACAGC CTGGGGGTC CTGCGTCTC TCCTGTGCG CCTCGGNTT CACCTTTTAG CGGTATGGGA  
 CTCACGTCG ACAACCTCAG ACCGCTCCG AACCATGTCG GACCCCCAG GAGCGAGAG AGGACACGTC GGAGGCTTAA GTGGAAATC GCCATACCT  
 V W V R Q A P G K G L E W V S A I S G S T Y Y A D S V K G R  
 TGTGTTGGT CCGCCAGCT CAGGGGAGG GTCTAGAGTG GGTCTAGCT ATTAGTGTA GTGTGTGAG CACATCTAC GCAGACTCG TGAAGGGCG  
 ACCAACCCTA GCGGTCCGA GTTCCCTTCC CAGATCTCAC CAGAGTCGA TAATCACCAT CACCACCATC GTGTATGATG CGTCTGAGGC ACTTCCCGC  
 F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y C A K R H  
 GTTCACATC TCCGCGACA ATTCCAAGAA CAGCTGTAT CTGCAATGA ACAGCTGCG TGCAGGAC ACCGCGGTAT ATTACTGTGC GAAAGGAT  
 CNAAGTGTAG AGGGCGCTGT TAAGTTCTT GTGCGACATA GAGTTTACT TGTGCGACGC ACGGCTCCTG TGGCGCCATA TAATGACACG CTTTCCCGTA  
 S S E A R Q F D Y W G Q G T L V T V S  
 AGTCTGAGG CTAGGCAATT TGACTACTGG GGCAGGGA CCTGTGTCAC CGTCTCG  
 TCAGACTCC GATCGTCAA ACTGATGACC CCGTCCCTT GGGACCAATG GCAGAGC

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